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(71) Applicant *(for all designated States except US)*: BIONOVA CORPORATION [US/US]; 1533 - 34th Avenue, San Francisco, CA 94122 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LIAO, Jaw-Ching [-/-]; 3F No.3 - 25 Valley, Chung-Cheng Road, Pan-Chiao, Taipei (TW). WANG, Cheng-nan [-/-]; 12th floor, No. 242, Kuang Fu South Road, Taipei (TW).

(74) Agents: KING, Joshua et al.; Seed and Berry L.L.P., 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).

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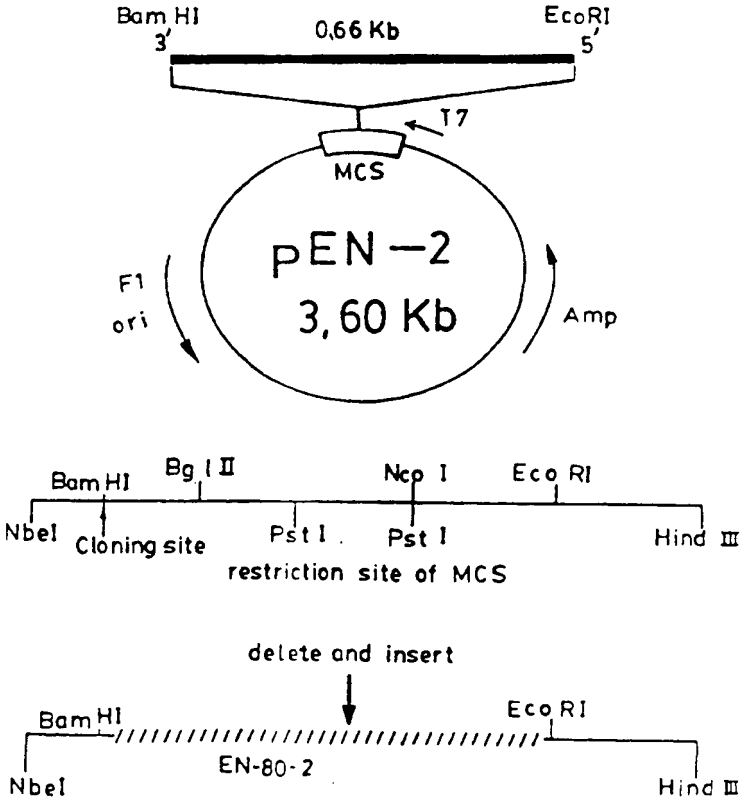
(54) Title: COMPOSITIONS AND METHODS FOR THE DIAGNOSIS OF, AND VACCINATION AGAINST, HEPATITIS C VIRUS (HCV)

(57) Abstract

The unprocessed core protein region initially translated from the genome of HCV contains epitopic configurations that are not retained in the processed proteins. In particular, the core protein loses an epitopic configuration upon processing at the cleavage site between the genomic region (e.g., gene) encoding the core protein and the genomic region encoding the adjacent envelope region. The unprocessed epitopic configuration of the core region provides an improved ability to detect the presence of HCV, or antibodies to HCV, in a sample, including an unpurified sample or a sample of very small volume (which can be particularly helpful when testing a sample from an infant or other person having very little blood (or other suitable material) available for testing). Combining the unprocessed core region with a non-structural protein (such as an NS5 or an NS3-NS4 fusion) results in a synergistic effect that greatly enhances the already improved sensitivity and specificity provided by the unprocessed core region. The unprocessed epitopic configuration of the core region also provides an improved ability to induce an immune response upon administration of the core region into an animal.



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Description

COMPOSITIONS AND METHODS FOR THE DIAGNOSIS OF, AND VACCINATION AGAINST, HEPATITIS C VIRUS (HCV)

Technical Field

The present invention relates generally to methods and compositions for the highly specific, highly sensitive diagnosis of Hepatitis C virus (HCV). The methods and compositions are also suitable for the elicitation of an immune response in an animal, and for the vaccination of an animal, against HCV.

Background Of The Invention

Most cases of hepatitis arising from blood transfusion are induced virally, and are distinguishable from other forms of viral-associated liver diseases caused by known hepatitis viruses such as hepatitis A virus (HAV) and hepatitis B virus (HBV). The etiological agent(s) of such Non-A, Non-B hepatitis (NANBH) has long been sought by many research groups and is presently believed to be the hepatitis C virus (HCV). Post-transfusion hepatitis (PTH) occurs in approximately 10% of transfusion patients, and NANBH accounts for up to 90% of these cases. A major problem arising from this disease is the frequent progression to chronic liver damage (25-55%). Therefore, the demand for sensitive, specific methods for detecting HCV in contaminated blood or blood products is significant.

The hepatitis C virus (HCV) was first identified by molecular cloning and characterization of its RNA genome by Choo et al. (*Science 244*:359-362, 1989). A detailed disclosure of the genome of HCV, and some cDNA sequences and polypeptides derived therefrom, as well as methodologies relating to such subject matter, is provided in EP 0 318 216 Al in the name of Chiron Corporation. As suggested in EP 0 318 216 Al, HCV may be a flavivirus or flavi-like virus. With respect to general morphology, a flavivirus contains a central nucleocapsid surrounded by a lipid bilayer. It is believed that hepatitis C virus protein is composed of structural proteins including a nucleocapsid (core) protein (C), two glycosylated envelope proteins (El, E2) and several nonstructural proteins (NS1-5).

EP 0 318 216 Al discloses a synthesized polypeptide, C100-3, which contains 363 virally-encoded amino acids that can be used for the detection of one type of HCV antibody. A specific assay using such HCV antigen designated C100-3 has been created, using recombinant DNA methods in yeast (*Science 244*:362-364).

Presently, kits for detecting HCV antibodies on the basis of the C100-3 antigen have been commercialized by Abbott Laboratories. It has been confirmed that C100-3 is a protein encoded by part of the nonstructural regions 3-4 of the HCV genome. However, it has been found that anti-C100-3 antibody is not detected in all post-transfusion NANBH cases. The failure to detect the anti-C100-3 antibody is possibly due to hypermutation of the nucleotide sequence in the C100-3 antigen.

In addition to the work with nonstructural C100-3 antigen, an enzyme-linked immunosorbent assay (ELISA) has been developed for serological diagnosis of hepatitis C virus (HCV) infection using the HCV core protein (p22). The core protein was synthesized by a recombinant baculovirus, as reported in Chiba et al. (*Proc. Natl. Acad. Sci. USA 88*:4641-4645, 1991). Thus, the assay of *Chiba, et al.* used a nonglycosylated 22-kDa nucleocapsid (core) protein, in an effort to establish an antibody-based, specific, sensitive method for diagnosing HCV infection. However, this core protein-based assay still failed to detect a significant number of cases of HCV infection, even when relatively large sample volumes were available.

Thus, there has gone unmet a need for compositions and methods capable of highly specific, highly sensitive detection of HCV. There has also gone unmet a need for compositions and methods capable of eliciting an immune response to HCV, especially an immunoprotective immune response to HCV. The present invention provides these and other related advantages.

Summary Of The Invention

The present invention is directed to the discovery that there are significant advantages in antigenicity and epitopic configuration in unprocessed polypeptides derived from HCV. These advantages are particularly useful for the detection and diagnosis of HCV, and also provide significantly enhanced compositions and methods for the induction of immune responses in an animal, and are expected to provide significantly enhanced vaccination of such an animal.

Accordingly, the present invention features an isolated polypeptide comprising an HCV core antigen protein joined to an amino-terminal portion of an HCV envelope region in unprocessed form, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV (this polypeptide is also referred to herein as an "unprocessed core antigen-envelope protein"). The present invention provides the first discovery that the epitopic configurations occur specifically in the

unprocessed core-envelope region, and surprisingly finds that these epitopic configurations can be found in an isolated protein.

Thus, in a first aspect the present invention provides an HCV-derived composition comprising, a) an isolated polypeptide comprising an HCV core antigen protein joined to an amino-terminal portion of an HCV envelope region in unprocessed form, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV; and b) an isolated HCV nonstructural protein. In one embodiment, the nucleic acid molecule is derived from a flavivirus or flavi-like virus other than HCV (other aspects of the invention disclosed herein are also suitable for use with such flavivirus or flavi-like virus).

In a preferred embodiment, the unprocessed core antigen-envelope protein is encoded by a nucleic acid molecule as set forth in SEQ ID No. 7. In other preferred embodiments for each of the features of the present invention, the isolated HCV nonstructural protein comprises an NS5 nonstructural protein or an NS3-NS4 unprocessed nonstructural protein. Further preferably, the NS5 nonstructural protein is encoded by a nucleic acid molecule as set forth in SEQ ID No. 9, and the NS3-NS4 unprocessed nonstructural protein is encoded by a nucleic acid molecule as described below.

In further embodiments, the unprocessed core antigen-envelope protein and/or nonstructural protein is produced by a suitable prokaryotic host cell, preferably a bacterium, and further preferably an *E. coli* BL21 (DE3). In alternative embodiments, the unprocessed core antigen-envelope protein and/or nonstructural protein is produced by a suitable eukaryotic host cell that is unable to process the isolated polypeptide.

In a further aspect, the present invention provides a method of making a composition comprising multiple polypeptides obtained from an HCV, comprising the following steps: a) introducing into a first host cell a first expression vector capable of expressing a nucleic acid molecule encoding an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV, b) incubating the first host cell under conditions suitable for the expression vector to produce the polypeptide, c) isolating the fusion polypeptide to provide an isolated fusion polypeptide, and also d) introducing into a second host cell a second expression vector capable of expressing a nucleic acid molecule encoding an isolated HCV nonstructural protein, e)incubating the second host cell under conditions suitable for the nucleic acid molecule to produce the HCV

nonstructural protein, f) isolating the HCV nonstructural protein to provide an isolated HCV nonstructural protein, and then g) combining the isolated fusion polypeptide and the isolated HCV nonstructural protein in the composition.

In a related aspect, the present invention provides a method of making a composition comprising multiple polypeptides obtained from an HCV, comprising the following steps: a) introducing into a suitable host cell an expression vector capable of expressing a first nucleic acid molecule encoding an isolated polypeptide comprising an HCV core antigen protein joined to an amino-terminal portion of an HCV envelope region in unprocessed form, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV, the expression vector further capable of expressing a second nucleic acid molecule encoding an HCV nonstructural protein, b) incubating the first host cell under conditions suitable for the expression vector to produce the polypeptide, and c) isolating the polypeptide and the HCV nonstructural protein.

In preferred embodiments, the unprocessed core antigen-envelope protein and/or the nonstructural protein are bound to a solid substrate. Further preferably, the protein(s) are covalently bound to the solid substrate.

In a further aspect, the present invention provides an assay for the detection of HCV in a sample, comprising: a) providing an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV, b) contacting the isolated polypeptide with the sample under conditions suitable and for a time sufficient for the polypeptide to bind to one or more antibodies present in the sample, to provide an antibody-bound polypeptide, and c) detecting the antibody-bound polypeptide, and therefrom determining that the sample contains HCV.

In a preferred embodiment, the assay further comprises: a) in step a), providing an HCV nonstructural protein bound to the solid substrate, b) in step b), contacting the HCV nonstructural protein with the sample under conditions suitable and for a time sufficient for the HCV nonstructural protein to bind to one or more antibodies present in the sample, to provide an antibody-bound HCV nonstructural protein, and c) in step c), detecting one or both of the antibody-bound polypeptide or the antibody-bound HCV nonstructural protein, and therefrom determining that the sample contains HCV.

In preferred embodiments for each of the aspects of the invention, the sample in the assay is an unpurified sample, preferably from an animal, and further preferably from a human being.

In other preferred embodiments, the assay is selected from the group consisting of a countercurrent immuno-electrophoresis (CIEP) assay, a radioimmunoassay, a radioimmunoprecipitation, an enzyme-linked immunosorbent assay (ELISA), a dot blot assay, an inhibition or competition assay, a sandwich assay, an immunostick (dip-stick) assays, a simultaneous assay, an immunochromatographic assay, an immunofiltration assay, a latex bead agglutination assay, an immunofluorescent assay, a biosensor assay, and a low-light detection assay. Still further, the assay is preferably not a western blot assay.

In another aspect, the present invention provides a method of producing an antibody, comprising the following steps: a) administering to an animal an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV under conditions suitable and for a time sufficient to induce an immune response in the animal to the polypeptide, thereby providing antibodies to the polypeptide, and b) isolating the antibodies to the polypeptide. The present invention also provides antibodies produced according to this method. Preferably, the antibodies are bound to a solid substrate.

In yet further aspect, the present invention provides an assay for the detection of HCV in a sample, comprising: a) contacting the sample with an antibody specific for an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV, under conditions suitable and for a time sufficient for the antibody to bind the unprocessed HCV core antigen protein, to provide a bound antibody, and b)detecting the bound antibody, and therefrom determining that the sample contains HCV.

Preferably, the assay additionally includes a) in step a), contacting the sample with a further antibody specific for an HCV nonstructural protein under conditions suitable and for a time sufficient for the further antibody to bind the HCV nonstructural protein, to provide a bound further antibody, and b) in step b), detecting one or both of the bound antibody or the bound further antibody, and therefrom determining that the sample contains HCV. Further preferably, the assay is selected from the group consisting of a countercurrent immuno-electrophoresis (CIEP) assay, a

radioimmunoassay, a radioimmunoprecipitation, an enzyme-linked immunosorbent assay (ELISA), a dot blot assay, an inhibition or competition assay, a sandwich assay, an immunostick (dip-stick) assays, a simultaneous assay, an immunochromatographic assay, an immunofiltration assay, a latex bead agglutination assay, an immunofluorescent assay, a biosensor assay, and a low-light detection assay. Even further preferably, the assay is not a western blot assay.

In still yet another aspect, the present invention provides a composition capable of eliciting an immune response in an animal comprising an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV, in combination with a pharmaceutically acceptable carrier or diluent. Preferably, the composition further comprises an HCV nonstructural protein.

In still yet a further aspect, the present invention provides a vaccine against HCV comprising an immunoprotective amount of an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV, in combination with a pharmaceutically acceptable carrier or diluent. Preferably, the vaccine further comprises an HCV nonstructural protein.

In another aspect, the present invention provides a method of inducing an immune response in an animal comprising administering to the animal a composition comprising an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV, in combination with a pharmaceutically acceptable carrier or diluent, under conditions suitable and for a time sufficient to induce the immune response. Preferably, the composition further comprises an HCV nonstructural protein.

In a related aspect, the present invention provides a method of vaccinating an animal comprising administering to the animal a composition comprising an immunoprotective amount of an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope

region of the HCV, in combination with a pharmaceutically acceptable carrier or diluent, under conditions suitable and for a time sufficient to induce an immunoprotective immune response. Preferably, the vaccine further comprises an HCV nonstructural protein.

In a further aspect, the present invention provides a kit for the detection of HCV comprising: a) an isolated polypeptide comprising an HCV core antigen protein joined to an amino-terminal portion of an envelope region of the HCV in unprocessed form, wherein the amino-terminal portion of the envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV, covalently bound to a solid substrate, and b)means for detecting the isolated polypeptide, particularly when the isolated polypeptide is bound to an antibody specific for HCV. Preferably, the kit further comprises an HCV nonstructural protein, and/or means for detecting the HCV nonstructural protein (particularly when bound to an antibody specific for HCV).

In a related aspect, the present invention provides a kit for the detection of HCV comprising: a) an antibody specific for an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV, under conditions suitable and for a time sufficient for the antibody to bind the unprocessed HCV core antigen protein, to provide a bound antibody, and b) means for detecting the antibody, particularly when bound to an antigen specific for HCV. Preferably, the kit further comprises a further antibody specific for an HCV nonstructural protein and means for detecting the further antibody (particularly when bound to an antigen specific for HCV).

In yet another further aspect, the present invention provides the compositions and vaccines described herein for use as a medicament to inhibit, prevent and/or treat HCV infection in an animal. In a related embodiment, the present invention provides such a composition for use in the manufacture of a medicament to inhibit, prevent or treat HCV infection in an animal. In preferred embodiments, the composition further comprises an HCV nonstructural protein, and/or is for use with a human being.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth throughout this specification that describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.); such references are incorporated by reference in their entirety.

Brief Description Of The Drawings

Fig. 1A depicts the nucleotide sequence of a nucleic acid molecule encoding a polypeptide comprising an HCV core antigen protein joined to an amino-terminal portion of an HCV envelope region in unprocessed form.

Fig. 1B depicts the amino acid sequence encoded by the nucleotide sequence depicted in Fig. 1A.

Fig. 2 shows the structure of the expression vector pEN-2, which was constructed by inserting a cDNA encoding an HCV core antigen protein joined to an amino-terminal portion of an HCV envelope region in unprocessed form into a plasmid. The figure also shows a restriction map illustrating certain significant features of the vector pEN-2.

Fig. 3A depicts the nucleotide sequence of a nucleic acid molecule encoding a polypeptide comprising an NS5 nonstructural region.

Fig. 3B depicts the amino acid sequence encoded by the nucleotide sequence depicted in Fig. 3A.

Fig. 4 shows the structure of the expression vector pEN-1, which was constructed by inserting a cDNA encoding an NS5 nonstructural region into a plasmid. The figure also shows a restriction map illustrating certain significant features of the vector pEN-1.

Detailed Description Of The Invention

The present invention is based on the discovery that the unprocessed core protein region as translated from the genome of HCV contains epitopic configurations that are not retained in the processed proteins. In particular, the core protein loses epitopic configuration(s) upon processing at the cleavage site between the genomic region (*e.g.*, gene) encoding the core protein and the genomic region encoding the adjacent envelope protein. As discussed below in the Examples portion of the present disclosure, the unprocessed epitopic configuration of the core region provides a surprisingly improved ability to detect the presence of HCV, or antibodies to HCV, in a sample, including an unpurified sample or a sample of very small volume (which can be particularly helpful when testing a sample from an infant or other person having very little blood (or other suitable material) available for testing).

Even more surprising, combining the unprocessed core region with a non-structural protein (such as an NS5 protein or an NS3-NS4 fusion protein) results in

a synergistic effect that greatly enhances the already improved sensitivity and specificity provided by the unprocessed core region.

These significant advantages in antigenicity and epitopic configuration also provide surprisingly enhanced compositions and methods for the induction of immune responses in an animal, enhanced vaccination of such an animal.

Accordingly, the present invention features an isolated polypeptide comprising an HCV core antigen protein joined to an amino-terminal portion of an HCV envelope region in unprocessed form, wherein the amino-terminal portion of the HCV envelope region is sized such that the polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of the HCV.

The present invention provides the first discovery that the lost epitopic configuration occurs in the core-envelope region, and surprisingly finds that the epitopic configuration can be found in an isolated protein.

By "core antigen protein" it is meant a polypeptide comprising the portion of the core protein that displays the antigenicity of the core protein. Although alteration of epitopic configuration upon processing was not known in the art, the core protein generally, and regions of the core protein that can be important to antigenicity, is well known in the art (*see, e.g.*, Okamoto et al., *J. Virol. 188*:331, 1992; Wang, U.S. Patent No. 5,106,726; Sallberg et al., *Immunology Letters 33*:27-34, 1992; Clemens, J.M. et al., *Blood 79*:1, 169-72, 1992; Houghton et al. (European Patent Applications 0,388,232 and 0,318,216); Harada et al., *J. Virol. 65*: 3015-3021 (1991); Hijikata et al., *P.N.A.S. 88*: 5547-5551 (1991)). Typically, it is the amino-terminal portion of the core protein that is important to the immunoreactivity of the core protein. A core antigen protein may be determined by SDS-PAGE and amino acid sequence analysis in light of the above references, and may also be determined by use in established HCV assays in light of the above references.

The present invention features this core antigen protein joined to an amino-terminal portion of an HCV envelope region in unprocessed form to supply the inventive "core-envelope fusion protein." In unprocessed form means that the core region and the envelope region are typically, and preferably, maintained precisely as they are joined (*i.e.*, encoded) in a native HCV. However, it is well within the skill of the art to make conservative amino acid substitutions, or insignificant amino acid additions, modifications or deletions, that may change the amino acid sequence of the core-envelope fusion protein but do not significantly alter the functioning of the protein (*i.e.*, the unprocessed epitopic configuration is retained). Such modifications may, however, when desired, delete the processing signal and/or site of the protein. These modifications are discussed further below.

The amino-terminal portion of the HCV envelope region is sized such that the fusion protein has an epitopic configuration specific to an unprocessed core-envelope region of said HCV. Thus, the amino-terminal portion of the HCV envelope region must be of sufficient length to permit the fusion protein to display the transient epitopic configuration specific to the unprocessed core region.

Determination of whether a given protein displays the epitopic configuration of the inventive core-envelope fusion protein can be performed as follows. A core-envelope protein in question may be included in a panel of core-envelope proteins that includes an established core-envelope fusion protein such as EN-80-2. The panel may be placed in a series of wells on a microtiter plate. The panel may also include other core-envelope proteins having different lengths of envelope protein. In a separate well is placed an established nonstructural protein capable of synergistic cooperation with the core-envelope fusion protein, such as EN-80-1. An antiserum is then selected for the established core-envelope fusion protein that reacts weakly with the established core-envelope fusion protein and that also is nonreactive with the established nonstructural protein. The basis for selection is that the antiserum will react with the separated proteins as expected, but the antiserum will react much more strongly when both a suitable core-envelope fusion protein and the established nonstructural protein are present in the sample. Preferably, the antiserum will react at least about 1.25 or 1.5 times as strongly as with the combined proteins when compared to the additive reaction of the antiserum with each protein, alone. Further preferably, the antiserum will react at least about twice as strongly. Many examples of such an antiserum, such as G614 (diluted 8-fold), G614 (diluted 16-fold), G615 (diluted 8-fold), G615 (diluted 16-fold), and 8-5, are set forth below in the Examples. The antiserum is introduced to the sample proteins under conditions suitable for elicitation and detection of an immune response between the antiserum and the given protein, and then such response is detected and measured. Next, the established nonstructural protein is combined with a further sample of each member of the core-envelope fusion protein panel. The antiserum is introduced to the combined proteins under conditions suitable for elicitation and detection of an immune response between the antiserum and the proteins, and such response is detected and measures. Those core-envelope proteins that provide a cooperative effect are suitable for use in the present invention. Each of the above-recited steps is routine in the art, in light of the present specification.

The core antigen-envelope fusion protein is preferably isolated, which means that the core antigen-envelope fusion protein is separated from the remainder of the polyprotein originally translated from the genome of HCV.

As noted above, in a highly preferred embodiment, the core antigen-envelope fusion protein of the present invention is used in combination with a nonstructural protein from HCV. The nonstructural coding region of HCV is well known in the art. *See, e.g.*, EP 0 318 216 Al.

The decision of which nonstructural protein, including portions of the nonstructural coding region that may include more than one nonstructural protein (or less than all of one nonstructural protein), can be made by selecting a nonstructural protein as follows. A nonstructural protein in question may be included in a panel of nonstructural proteins that includes an established nonstructural protein such as EN-80-1. The panel may be placed in a series of wells on a microtiter plate. The panel may also include other nonstructural proteins. In a separate well is placed an established core-envelope fusion protein capable of synergistic cooperation with the nonstructural protein, such as EN-80-2. An antiserum is selected for the established core-envelope fusion protein that reacts weakly with the established core-envelope fusion protein and that also is nonreactive with the established nonstructural protein. The basis for selection is that the antiserum will react with the separated proteins as expected, but the antiserum will react much more strongly when both the established core-envelope fusion protein and a suitable nonstructural protein are present in the sample. As set forth above, many examples of such an antiserum are set forth below in the Examples. The antiserum is introduced to the separated sample proteins under conditions suitable for elicitation and detection of an immune response between the antiserum and the given protein, and then such response is detected and measured. The established core-envelope protein is combined with each member of the nonstructural protein panel. Next, the antiserum is introduced to the combined proteins under conditions suitable for elicitation and detection of an immune response between the antiserum and the proteins, and then such response is detected and measured. Those nonstructural proteins that provide a cooperative effect are suitable for use in the present invention. Each of the above-recited steps is routine in the art, in light of the present specification.

The present invention also provides antibodies, preferably monoclonal antibodies, to the core-envelope fusion protein and/or the nonstructural protein, as well as other proteins of the present invention. The antibodies are preferably used in combination to provide particularly sensitive and specific detection of HCV in a sample.

Still further, the present invention provides compositions and methods for the elicitation on an immune response in an animal (either humoral, cellular, or both). Even further, the compositions and methods can vaccinate an animal against HCV.

Preferably, the methods and compositions of the present invention, including those for detection, immune response elicitation and vaccination, are applied to a human being.

Nucleic Acid Molecules Encoding An HCV Core Antigen Protein Joined To An Amino-Terminal Portion Of An HCV Envelope Region In Unprocessed Form And/Or A Nonstructural Protein Of The Invention

As noted above, the present invention provides a nucleic acid molecule encoding a polypeptide comprising an HCV core antigen protein joined to an amino-terminal portion of an HCV envelope region in unprocessed form. The present invention also provides a nucleic acid molecule encoding a polypeptide comprising a nonstructural protein of HCV. In a preferred embodiment, the nucleic acid molecule is DNA.

In one preferred embodiment, the nucleic acid molecule is a DNA molecule that encodes an unprocessed core antigen-envelope protein that was isolated from nucleic acid sequences present in the plasma of an HCV infected patient. As discussed further below, the isolation of the DNA included the steps of isolating viral particles from the patient's plasma, extracting and purifying the viral nucleic acid sequences, and then cloning the desired DNA molecule via a Polymerase Chain Reaction (PCR) technique. The primers used for cloning were as follows:

(i) 5'-GGATCCATGAGCACAAATCCTAAACCT-3' (SEQ ID No. 1)

and

(ii) 5'-GAATTCGGTGTGCATGATCATGTCCGC-3' (SEQ ID No. 2).

The cloned DNA molecule was sequenced in order to confirm its identity. The molecule thus obtained was designated EN-80-2. The DNA sequence of the molecule EN-80-2 is given in Fig. 1A (SEQ ID No. 7), and has 669 bp. The amino acid sequence of the molecule EN-80-2 is given in Fig. 1B (SEQ ID No. 8), and has 223 residues. The molecule EN-80-2, in *E. coli* strain BL21(DE3), was deposited with the American Type Culture Collection (ATCC) Rockville Maryland 20852, on July 14, 1993, and has been accorded ATCC Designation 55451. The culture has been deposited under the conditions of the Budapest Treaty.

In another preferred embodiment, the nucleic acid molecule is a DNA molecule encoding an HCV NS5 nonstructural protein that was isolated from nucleic

acid sequences present in the plasma of an HCV infected patient. As with the isolation of the unprocessed core antigen-envelope protein discussed above (although conducted with a different patient), the isolation included the steps of isolating viral particles from the patient's plasma, extracting and purifying the viral nucleic acid sequences, and then cloning the desired DNA molecule via a Polymerase Chain Reaction (PCR) technique. The primers used in the PCR were as follows:

(i) 5'-GGATCCCGGTGGAGGATGAGAGGGAAATATCCG-3' (SEQ ID No. 3) and

(ii) 5'-GAATTCCCGGACGTCCTTCGCCCCGTAGCCAAATTT-3' (SEQ ID No. 4)

The isolated DNA molecule was subjected to sequence analysis in order to confirm its identity. The molecule thus obtained was designated EN-80-1. The DNA sequence of the molecule EN-80-1 is given in Fig. 3A (SEQ ID No. 9) and has 803 bp. The amino acid sequence of the molecule EN-80-1 is given in Fig. 3B (SEQ ID No. 10), and has 267 residues. The molecule EN-80-1, in *E. coli* strain BL21(DE3), was deposited with the American Type Culture Collection (ATCC) Rockville Maryland 20852, on July 14, 1993, and has been accorded ATCC Designation 55450. The culture has been deposited under the conditions of the Budapest Treaty.

This general procedure has also been used to isolate a representative nucleic acid molecule from the NS3-NS4 nonstructural region of HCV. *See also* Simmonds, *Lancet 336*: 1469-1472, 1990. The primers used for the cloning were as follows:

(i ("ED3")) 5'-CACCCAGACAGTCGATTTCAG-3' (SEQ ID No. 5) and
(ii ("ED4")) 5'-GTATTTGGTGACTGGGTGCGTC-3' (SEQ ID No. 6)

The molecule thus obtained was designated EN-80-4. The polypeptide encoded by the isolated molecule has a molecular weight of about 20,000 Daltons as measure by electrophoresis through SDS-PAGE.

The nucleic acid molecules of the present invention, such as those described above, can be inserted into an appropriate vector to form an expression vector. Figure 2 depicts an expression plasmid, pEN-2, that contains the DNA molecule encoding the unprocessed core antigen-envelope protein isolated using the primers SEQ ID No. 1 and 2, discussed above. Figure 4 depicts an expression plasmid, pEN-1, that

contains the DNA molecule encoding the NS5 nonstructural protein isolated using the primers SEQ ID No. 3 and 4, discussed above.

The present invention provides for the manipulation and expression of the above described nucleic acid molecules by culturing host cells containing a construct capable of expressing the above-described genes.

Numerous vector constructs suitable for use with the nucleic acid molecules of the present invention can be prepared as a matter of convenience. Within the context of the present invention, a vector construct is understood to typically refer to a DNA molecule, or a clone of such a molecule (either single-stranded or double-stranded), that has been modified through human intervention to contain segments of DNA combined and juxtaposed in a manner that as a whole would not otherwise exist in nature. Vector constructs of the present invention comprise a first DNA segment encoding one or more of an unprocessed core antigen-envelope protein and a nonstructural protein of HCV operably linked to additional DNA segments required for the expression of the first DNA segment. Within the context of the present invention, additional DNA segments will typically include a promoter and will generally include transcription terminators, and may further include enhancers and other elements. *See* WO 94/25597 and WO/25598.

Mutations in nucleotide sequences constructed for expression of the inventive proteins preferably preserve the reading frame of the encoding sequences. Furthermore, the mutations will preferably not create complementary regions that could hybridize to produce secondary mRNA structures, such as loops or hairpins, that would adversely affect translation of the mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the mutation *per se* be predetermined. For example, in order to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at the target codon and the expressed mutants screened for indicative biological activity.

Mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes a derivative having the desired amino acid insertion, substitution or deletion.

Alternatively, oligonucleotide-directed, site-specific mutagenesis procedures may be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene 42*:133, 1986); Bauer et al. (*Gene 37*:73, 1985); Craik (*BioTechniques*, January 1985, 12-19);

Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); and Sambrook et al. (*supra*).

The primary amino acid structure of the above described proteins, may also be modified by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups, or with other proteins or polypeptides, provided that such modifications should not interfere with the antigenicity of the proteins. (*See* U.S. Patent No. 4,851,341; *see also* Hopp et al., *Bio/Technology* 6:1204, 1988). For example, such modifications should not interfere with the epitopic configuration (including access to the epitope and other antigenic considerations) specific to the unprocessed core antigen-envelope protein.

A preferred type of vector construct is known as an expression vector. As noted above, the plasmids pEN-1 and pEN-2 are examples of such expression vectors, and contain nucleic acid molecules encoding the NS5 nonstructural region and the unprocessed core antigen-envelope protein, respectively.

For expression, a DNA molecule as described above is inserted into a suitable vector construct, which in turn is used to transform or transfect appropriate host cells for expression. Preferably, the host cell for use in expressing the gene sequences of the present invention is a prokaryotic host cell, further preferably a bacterium such as *E. coli*. Other suitable host cells include *Salmonella, Bacillus, Shigella, Pseudomonas, Streptomyces* and other genera known in the art. In a further preferred embodiment, the host cell is an *E. coli* containing a DE3 lysogen or T7 RNA polymerase, such as BL21(DE3), JM109(DE3) or BL21(DE3) pLysS.

Vectors used for expressing cloned DNA sequences in bacterial hosts will generally contain a selectable marker, such as a gene for antibiotic resistance, and will contain a promoter that functions in the host cell. Appropriate promoters include the *trp* (Nichols and Yanofsky, *Meth. Enzymol. 101*:155-164, 1983), *lac* (Casadaban et al., *J. Bacteriol. 143*:971-980, 1980), and phage λ (Queen, *J. Mol. Appl. Genet. 2*:1-10, 1983) promoter systems. The expression units may also include a transcriptional terminator. Plasmids useful for transforming bacteria include the pUC plasmids (Messing, *Meth. Enzymol. 101*:20-78, 1983; Vieira and Messing, *Gene 19*:259-268, 1982), pBR322 (Bolivar et al., *Gene 2*:95-113, 1977), pCQV2 (Queen, *ibid.*), and derivatives thereof. Plasmids may contain both viral and bacterial elements.

In another embodiment, the host cell may be a eukaryotic cell, provided that either the host cell has been modified such that the host cell cannot process, for example, the unprocessed core antigen-envelope protein or unprocessed nonstructural region (such as the NS3-NS4 nonstructural protein), or the processing signals and/or processing sites in the unprocessed protein have been modified such that the protein is

no longer susceptible to processing (such modifications should not affect the antigenicity of the unprocessed protein). Eukaryotic host cells suitable for use in practicing the present invention include mammalian, avian, plant, insect and fungal cells. Preferred eukaryotic cells include cultured mammalian cell lines (*e.g.*, rodent or human cell lines), insect cell line (*e.g.*, Sf-9) and fungal cells, including species of yeast (*e.g.*, *Saccharomyces* spp., particularly *S. cerevisiae*, *Schizosaccharomyces* spp., or *Kluyveromyces* spp.) or filamentous fungi (*e.g.*, *Aspergillus* spp., *Neurospora* spp.).

Techniques for transforming these host cells, and methods of expressing foreign DNA sequences cloned therein, are well known in the art (*see*, *e.g.*, Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1982; Sambrook et al., *supra;* "Gene Expression Technology," *Methods in Enzymology*, Vol. 185, Goeddel (ed.), Academic Press, San Diego, Calif., 1990; "Guide to Yeast Genetics and Molecular Biology," *Methods in Enzymology*, Guthrie and Fink (eds.), Academic Press, San Diego, Calif., 1991; Hitzeman et al., *J. Biol. Chem. 255*:12073-12080, 1980; Alber and Kawasaki, *J. Mol. Appl. Genet. 1*:419-434, 1982; Young et al., in *Genetic Engineering of Microorganisms for Chemicals*, Hollaender et al. (eds.), p. 355, Plenum, New York, 1982; Ammerer, *Meth. Enzymol. 101*:192-201, 1983; McKnight et al., U.S. Patent No. 4,935,349).

In general, a host cell will be selected on the basis of its ability to produce the protein of interest at a high level. In this way, the number of cloned DNA sequences that must be introduced into the host cell can be minimized and overall yield of biologically active protein can be maximized. Given the teachings provided herein, promoters, terminators and methods for introducing such expression vectors encoding the proteins of the present invention into desired host cells would be evident to those of skill in the art.

Host cells containing vector constructs of the present invention are then cultured to express a DNA molecule as described above. The cells are cultured according to standard methods in a culture medium containing nutrients required for growth of the chosen host cells. A variety of suitable media are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals, as well as other components, *e.g.*, growth factors or serum, that may be required by the particular host cells. The growth medium will generally select for cells containing the DNA construct(s) by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct.

Polypeptides Comprising An Unprocessed Core Antigen-Envelope Protein And/Or A Nonstructural Protein Of The Invention

As noted above, the invention provides a polypeptide comprising the HCV core antigen protein joined to an amino-terminal portion of an HCV envelope region in unprocessed form. In a preferred embodiment, the amino acid sequence of the polypeptide is that given in Fig. 1B (Seq. I.D. No. 8). In such a preferred embodiment, the polypeptide has a molecular weight of about 25,000 daltons as measured by electrophoresis through a sodium dodecyl sulfate-polyacrylamide gel and has been deduced to have about 223 amino acids.

The unprocessed core antigen-envelope protein is capable of binding HCV antibodies, which has been confirmed by Western Blotting and by enzyme-linked immunosorbent assay (ELISA). The unprocessed core antigen-envelope protein is specifically reactive with the sera of patients with HCV, and therefore is not significantly reactive with the sera of persons without HCV. The unprocessed core antigen-envelope protein is also capable of detecting the presence of HCV antibodies in samples, and therefore is useful for diagnosis of HCV in a patient, particularly a human being.

The present invention also provides a polypeptide comprising an HCV nonstructural protein. In a preferred embodiment, the polypeptide has the amino acid sequence of the polypeptide depicted in Fig. 3B (SEQ ID No. 10). The polypeptide of Figure 3B (SEQ ID No. 10) has a molecular weight of about 29,000 daltons as measured by electrophoresis through a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and has been deduced to have about 267 amino acids.

The nonstructural protein of the present invention is capable of binding HCV antibodies, which has been confirmed by Western Blotting and by enzyme-linked immunosorbent assay (ELISA) for both the NS5 and the NS3-NS4 nonstructural proteins disclosed herein. The nonstructural protein of the present invention is specifically reactive with the sera of patients with HCV, and therefore is not reactive with the sera of persons without HCV. The nonstructural protein is also capable of detecting the presence of HCV antibodies in samples, and therefore is useful for diagnosis of HCV in a patient, particularly a human being.

Where the protein of the present invention is encoded by a portion of a native gene, a derivative of a native gene, or has been otherwise modified, the protein maintains substantially the same biological activity of the native protein. For example, the structure of proteins corresponding to the unprocessed core antigen-envelope protein or the nonstructural protein can be predicted from the primary translation products thereof using the hydrophobicity plot function of, for example, P/C Gene or

Intelligenetics Suite (Intelligenetics, Mountain View, Calif.), or according to the methods described by Kyte and Doolittle (*J. Mol. Biol. 157*:105-132, 1982).

In a preferred embodiment, the present invention provides isolated proteins. Proteins can be isolated by, among other methods, culturing suitable host and vector systems to produce the recombinant translation products of the present invention. Supernatants from such cell lines, or protein inclusions or whole cells where the protein is not excreted or secreted into the supernatant, can then be treated by a variety of purification procedures in order to isolate the desired proteins. For example, the supernatant may be first concentrated using commercially available protein concentration filters, such as an Amicon or Millipore Pellicon ultrafiltration unit. Following concentration, the concentrate may be applied to a suitable purification matrix such as, for example, an anti-protein antibody bound to a suitable support. Alternatively, anion or cation exchange resins may be employed in order to purify the protein. As a further alternative, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps may be employed to further purify the protein. Other methods of isolating the proteins of the present invention are well known in the skill of the art.

A protein is deemed to be "isolated" within the context of the present invention if the protein accounts for at least about 90% of the protein, by weight, in a mixture. Within other embodiments, the desired protein can be isolated such that no other (undesired) protein, and preferably no lipopolysaccharide (LPS), is detected by SDS-PAGE analysis followed by coomassie blue staining, further preferably by SDS-PAGE analysis followed by silver staining. Within still other embodiments, the protein is isolated if no other protein having significant antigenic activity that significantly interferes with detection assays or immunological events is included with the protein.

Binding Partners To An Unprocessed Core Antigen-Envelope Protein Or A Nonstructural Protein Of The Invention

The present invention also provides monoclonal and polyclonal antibodies directed against the unprocessed core antigen-envelope protein or the nonstructural protein of HCV. The antibodies are produced by using the polypeptide of the invention as an immunogen through standard procedures for preparing a hybridoma, and/or other methods. The resulting antibodies are particularly useful for detecting HCV in a sample, preferably a sample from a human being.

Polyclonal antibodies can be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, turkeys, rabbits, mice, or rats. Briefly, the desired protein or peptide is

utilized to immunize the animal, typically through intraperitoneal, intramuscular, intraocular, or subcutaneous injections. The immunogenicity of the protein or peptide of interest may be increased through the use of an adjuvant such as Freund's complete or incomplete adjuvant. Following several booster immunizations, small samples of serum are collected and tested for reactivity to the desired protein or peptide.

Once the titer of the animal has reached a plateau in terms of its reactivity to the protein, larger quantities of polyclonal antisera may be readily obtained either by weekly bleedings, or by exsanguinating the animal.

Monoclonal antibodies can also be readily generated using well-known techniques (*see* U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993; *see also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and *Antibodies: A Laboratory Manual, supra*). Briefly, in one embodiment, a subject animal such as a rat or mouse is injected with a desired protein or peptide. If desired, various techniques may be utilized in order to increase the resultant immune response generated by the protein, in order to develop greater antibody reactivity. For example, the desired protein or peptide may be coupled to another protein such as ovalbumin or keyhole limpet hemocyanin (KLH), or through the use of adjuvants such as Freund's complete or incomplete adjuvants. The initial elicitation of an immune response may be through intraperitoneal, intramuscular, intraocular, or subcutaneous routes.

Between one and three weeks after the initial immunization, the animal may be reimmunized with booster immunization. The animal may then be test bled and the serum tested for binding to the unprocessed core antigen-envelope region, or to an HCV nonstructural protein using assays as described above. Additional immunizations may also be accomplished until the animal has reached a plateau in its reactivity to the desired protein or peptide. The animal may then be given a final boost of the desired protein or peptide, and three to four days later sacrificed. At this time, the spleen and lymph nodes may be harvested and disrupted into a single cell suspension by passing the organs through a mesh screen or by rupturing the spleen or lymph node membranes which encapsulate the cells. Within one embodiment the red cells are subsequently lysed by the addition of a hypotonic solution, followed by immediate return to isotonicity.

Within another embodiment, suitable cells for preparing monoclonal antibodies are obtained through the use of *in vitro* immunization techniques. Briefly, an animal is sacrificed, and the spleen and lymph node cells are removed as described above. A single cell suspension is prepared, and the cells are placed into a culture containing a form of the protein or peptide of interest that is suitable for generating an

immune response as described above. Subsequently, the lymphocytes are harvested and fused as described below.

Cells that are obtained through the use of *in vitro* immunization or from an immunized animal as described above may be immortalized by transfection with a virus such as the Epstein-Barr Virus (EBV). (*See* Glasky and Reading, *Hybridoma 8(4)*:377-389, 1989.) Alternatively, within a preferred embodiment, the harvested spleen and/or lymph node cell suspensions are fused with a suitable myeloma cell in order to create a "hybridoma" which secretes monoclonal antibodies. Suitable myeloma lines are preferably defective in the construction or expression of antibodies, and are additionally syngeneic with the cells from the immunized animal. Many such myeloma cell lines are well known in the art and may be obtained from sources such as the American Type Culture Collection (ATCC), Rockville, Maryland (*see Catalogue of Cell Lines & Hybridomas*, 6th ed., ATCC, 1988). Representative myeloma lines include: for humans, UC 729-6 (ATCC No. CRL 8061), MC/CAR-Z2 (ATCC No. CRL 8147), and SKO-007 (ATCC No. CRL 8033); for mice, SP2/0-Ag14 (ATCC No. CRL 1581), and P3X63Ag8 (ATCC No. TIB 9); and for rats, Y3-Ag1.2.3 (ATCC No. CRL 1631), and YB2/0 (ATCC No. CRL 1662). Particularly preferred fusion lines include NS-1 (ATCC No. TIB 18) and P3X63 - Ag 8.653 (ATCC No. CRL 1580), which may be utilized for fusions with either mouse, rat, or human cell lines. Fusion between the myeloma cell line and the cells from the immunized animal can be accomplished by a variety of methods, including the use of polyethylene glycol (PEG) (*see Antibodies: A Laboratory Manual, supra*) or electrofusion (*see* Zimmerman and Vienken, *J. Membrane Biol. 67*:165-182, 1982).

Following the fusion, the cells are placed into culture plates containing a suitable medium, such as RPMI 1640 or DMEM (Dulbecco's Modified Eagles Medium, JRH Biosciences, Lenexa, Kan.). The medium may also contain additional ingredients, such as Fetal Bovine Serum (FBS, *e.g.*, from Hyclone, Logan, Utah, or JRH Biosciences), thymocytes that were harvested from a baby animal of the same species as was used for immunization, or agar to solidify the medium. Additionally, the medium should contain a reagent which selectively allows for the growth of fused spleen and myeloma cells. Particularly preferred is the use of HAT medium (hypoxanthine, aminopterin, and thymidine) (Sigma Chemical Co., St. Louis, Mo.). After about seven days, the resulting fused cells or hybridomas may be screened in order to determine the presence of antibodies which recognizes the core-envelope region of said HCV or the HCV nonstructural protein. Following several clonal dilutions and reassays, hybridoma producing antibodies that bind to the protein of interest can be isolated.

Other techniques can also be utilized to construct monoclonal antibodies. (*See* Huse et al., "Generation of a Large Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," *Science 246*:1275-1281, 1989; *see also* Sastry et al., "Cloning of the Immunological Repertoire in *Escherichia coli* for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain Variable Region-Specific cDNA Library," *Proc. Natl. Acad. Sci. USA 86*:5728-5732, 1989; *see also* Alting-Mees et al., "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas," *Strategies in Molecular Biology 3*:1-9, 1990; these references describe a commercial system available from Stratacyte, La Jolla, California, which enables the production of antibodies through recombinant techniques.) Briefly, mRNA is isolated from a B cell population and utilized to create heavy and light chain immunoglobulin cDNA expression libraries in the λIMMUNOZAP(H) and λIMMUNOZAP(L) vectors. These vectors may be screened individually or co-expressed to form Fab fragments or antibodies (*see* Huse et al., *supra*; *see also* Sastry et al., *supra*). Positive plaques can subsequently be converted to a non-lytic plasmid which allows high level expression of monoclonal antibody fragments from *E. coli*.

Similarly, binding partners can also be constructed utilizing recombinant DNA techniques to incorporate the variable regions of a gene that encodes a specifically binding antibody. The construction of these binding partners can be readily accomplished by one of ordinary skill in the art given the disclosure provided herein. (*See* Larrick et al., "Polymerase Chain Reaction Using Mixed Primers: Cloning of Human Monoclonal Antibody Variable Region Genes From Single Hybridoma Cells," *Biotechnology 7*:934-938, 1989; Riechmann et al., "Reshaping Human Antibodies for Therapy," *Nature 332*:323-327, 1988; Roberts et al., "Generation of an Antibody with Enhanced Affinity and Specificity for its Antigen by Protein Engineering," *Nature 328*:731-734, 1987; Verhoeyen et al., "Reshaping Human Antibodies: Grafting an Antilysozyme Activity," *Science 239*:1534-1536, 1988; Chaudhary et al., "A Recombinant Immunotoxin Consisting of Two Antibody Variable Domains Fused to *Pseudomonas* Exotoxin," *Nature 339*:394-397, 1989; *see also* U.S. Patent No. 5,132,405 entitled "Biosynthetic Antibody Binding Sites".) Briefly, in one embodiment, DNA segments encoding the desired protein or peptide interest-specific antigen binding domains are amplified from hybridomas that produce a specifically binding monoclonal antibody, and are inserted directly into the genome of a cell that produces human antibodies. (*See* Verhoeyen et al., *supra*; *see also* Reichmann et al., *supra*.) This technique allows the antigen-binding site of a specifically binding mouse or rat monoclonal antibody to be transferred into a human antibody. Such antibodies are

preferable for therapeutic use in humans because they are not as antigenic as rat or mouse antibodies.

In an alternative embodiment, genes that encode the variable region from a hybridoma producing a monoclonal antibody of interest are amplified using oligonucleotide primers for the variable region. These primers may be synthesized by one of ordinary skill in the art, or may be purchased from commercially available sources. For instance, primers for mouse and human variable regions including, among others, primers for V_{Ha}, V_{Hb}, V_{Hc}, V_{Hd}, C_{H1}, V_L and C_L regions, are available from Stratacyte (La Jolla, Calif.). These primers may be utilized to amplify heavy or light chain variable regions, which may then be inserted into vectors such as IMMUNOZAP™(H) or IMMUNOZAP™(L) (Stratacyte), respectively. These vectors may then be introduced into *E. coli* for expression. Utilizing these techniques, large amounts of a single-chain protein containing a fusion of the V_H and V_L domains may be produced (*see* Bird et al., *Science 242*:423-426, 1988).

Monoclonal antibodies and binding partners can be produced in a number of host systems, including tissue cultures, bacteria, eukaryotic cells, plants and other host systems known in the art.

Once suitable antibodies or binding partners have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art (*see Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; U.S. Patent No. 4,736,110; and U.S. Patent No. 4,486,530). Suitable isolation techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques. Within the context of the present invention, the term "isolated" as used to define antibodies or binding partners means "substantially free of other blood components."

The antibodies and binding partners of the present invention have many uses. As discussed further below, the antibodies and binding partners of the present invention are particularly useful for the detection and diagnosis of HCV. Other uses include, for example, flow cytometry to sort cells displaying one more of the proteins of the present invention. Briefly, in order to detect the protein or peptide of interest on cells, the cells are incubated with a labeled monoclonal antibody which specifically binds to the protein of interest, followed by detection of the presence of bound antibody. These steps may also be accomplished with additional steps such as washings to remove unbound antibody. Labels suitable for use within the present invention are well known in the art including, among others, flourescein isothiocyanate (FITC), phycoerythrin (PE), horse radish peroxidase (HRP), and colloidal gold. Particularly preferred for use

in flow cytometry is FITC, which may be conjugated to purified antibody according to the method of Keltkamp in "Conjugation of Fluorescein Isothiocyanate to Antibodies. I. Experiments on the Conditions of Conjugation," *Immunology 18*:865-873, 1970. (*See also* Keltkamp, "Conjugation of Fluorescein Isothiocyanate to Antibodies. II. A Reproducible Method," *Immunology 18*:875-881, 1970; Goding, "Conjugation of Antibodies with Fluorochromes: Modification to the Standard Methods," *J. Immunol. Methods 13*:215-226, 1970.)

Assays For The Detection Of HCV In A Sample

The present invention provides methods for detecting HCV in a sample. The assays are typically based on the detection of antigens displayed by HCV or antibodies produced against HCV, but may also include nucleic acid based assays (typically based upon hybridization), as known in the art. The methods are characterized by the ability of the polypeptides of the present invention to be bound by anti-HCV antibodies, and the ability of antibodies produced against the proteins of the present invention to bind to HCV antigens in a sample.

Surprisingly, the unprocessed core antigen-envelope protein of HCV of the present invention provides significantly better and more sensitive detection of HCV in a sample than processed core protein (sometimes referred to as p22) or fragments of the core protein, alone. Even further surprisingly, the use of both unprocessed core antigen-envelope protein and a nonstructural protein of HCV in the assay provides a synergistic effect that permits significantly more sensitive detection of HCV than when either the unprocessed core antigen-envelope protein or nonstructural protein of HCV is utilized alone.

A preferred assay for the detection of HCV is a sandwich assay such as an enzyme-linked immunosorbent assay (ELISA). One preferred ELISA comprises the following steps: (1) coating a core antigen-envelope protein of the present invention onto a solid phase, (2) incubating a sample suspected of containing HCV antibodies with the polypeptide coated onto the solid phase under conditions that allow the formation of an antigen-antibody complex, (3) adding an anti-antibody (such as anti-IgG) conjugated with a label to be captured by the resulting antigen-antibody complex bound to the solid phase, and (4) measuring the captured label and determining therefrom whether the sample has HCV antibodies.

Although a preferred assay is set forth above, a variety of assays can be utilized in order to detect antibodies that specifically bind to the desired protein from a sample, or to detect the desired protein bound to one or more antibodies from the sample. Exemplary assays are described in detail in *Antibodies: A Laboratory Manual,*

Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: countercurrent immuno-electrophoresis (CIEP), radioimmunoassays, radioimmunoprecipitations, enzyme-linked immunosorbent assays (ELISA), dot blot assays, inhibition or competition assays, sandwich assays, immunostick (dip-stick) assays, simultaneous assays, immunochromatographic assays, immunofiltration assays, latex bead agglutination assays, immunofluorescent assays, biosensor assays, and low-light detection assays (*see* U.S. Patent Nos. 4,376,110 and 4,486,530; *see also Antibodies: A Laboratory Manual, supra*).

A fluorescent antibody test (FA-test) uses a fluorescently labeled antibody able to bind to one of the proteins of the invention. For detection, visual determinations are made by a technician using fluorescence microscopy, yielding a qualitative result. In one embodiment, this assay is used for the examination of tissue samples or histological sections.

In latex bead agglutination assays, antibodies to one or more of the proteins of the present invention are conjugated to latex beads. The antibodies conjugated to the latex beads are then contacted with a sample under conditions permitting the antibodies to bind to desired proteins in the sample, if any. The results are then read visually, yielding a qualitative result. In one embodiment, this format can be used in the field for on-site testing.

Enzyme immunoassays (EIA) include a number of different assays able to utilize the antibodies provided by the present invention. For example, a heterogeneous indirect EIA uses a solid phase coupled with an antibody of the invention and an affinity purified, anti-IgG immunoglobulin preparation. Preferably, the solid phase is a polystyrene microtiter plate. The antibodies and immunoglobulin preparation are then contacted with the sample under conditions permitting antibody binding, which conditions are well known in the art. The results of such an assay can be read visually, but are preferably read using a spectrophotometer, such as an ELISA plate reader, to yield a quantitative result. An alternative solid phase EIA format includes plastic-coated ferrous metal beads able to be moved during the procedures of the assay by means of a magnet. Yet another alternative is a low-light detection immunoassay format. In this highly sensitive format, the light emission produced by appropriately labeled bound antibodies are quantitated automatically. Preferably, the reaction is performed using microtiter plates.

In an alternative embodiment, a radioactive tracer is substituted for the enzyme mediated detection in an EIA to produce a radioimmunoassay (RIA).

In a capture-antibody sandwich enzyme assay, the desired protein is bound between an antibody attached to a solid phase, preferably a polystyrene microtiter

plate, and a labeled antibody. Preferably, the results are measured using a spectrophotometer, such as an ELISA plate reader. This assay is one preferred embodiment for the present invention.

In a sequential assay format, reagents are allowed to incubate with the capture antibody in a step wise fashion. The test sample is first incubated with the capture antibody. Following a wash step, an incubation with the labeled antibody occurs. In a simultaneous assay, the two incubation periods described in the sequential assay are combined. This eliminates one incubation period plus a wash step.

A dipstick/immunostick format is essentially an immunoassay except that the solid phase, instead of being a polystyrene microtiter plate, is a polystyrene paddle or dipstick. Reagents are the same and the format can either be simultaneous or sequential.

In a chromatographic strip test format, a capture antibody and a labeled antibody are dried onto a chromatographic strip, which is typically nitrocellulose or nylon of high porosity bonded to cellulose acetate. The capture antibody is usually spray dried as a line at one end of the strip. At this end there is an absorbent material that is in contact with the strip. At the other end of the strip the labeled antibody is deposited in a manner that prevents it from being absorbed into the membrane. Usually, the label attached to the antibody is a latex bead or colloidal gold. The assay may be initiated by applying the sample immediately in front of the labeled antibody.

Immunofiltration/immunoconcentration formats combine a large solid phase surface with directional flow of sample/reagents, which concentrates and accelerates the binding of antigen to antibody. In a preferred format, the test sample is preincubated with a labeled antibody then applied to a solid phase such as fiber filters or nitrocellulose membranes or the like. The solid phase can also be precoated with latex or glass beads coated with capture antibody. Detection of analyte is the same as standard immunoassay. The flow of sample/reagents can be modulated by either vacuum or the wicking action of an underlying absorbent material.

A threshold biosensor assay is a sensitive, instrumented assay amenable to screening large numbers of samples at low cost. In one embodiment, such an assay comprises the use of light addressable potentiometric sensors wherein the reaction involves the detection of a pH change due to binding of the desired protein by capture antibodies, bridging antibodies and urease-conjugated antibodies. Upon binding, a pH change is effected that is measurable by translation into electrical potential (μvolts). The assay typically occurs in a very small reaction volume, and is very sensitive. Moreover, the reported detection limit of the assay is 1,000 molecules of urease per minute.

Compositions And Methods For The Elicitation Of An Immune Response To HCV

The present invention also provides compositions and methods for the elicitation of an immune response to HCV, which may be either humoral, cellular, or both. Preferably, the immune response is induced by a vaccine against HCV, and is therefore an immunoprotective immune response. These compositions and methods typically involve an immunogen comprising an unprocessed core antigen-envelope protein or nonstructural protein of HCV in combination with a pharmaceutically acceptable carrier or diluent. In a preferred embodiment, the compositions and methods comprise both an unprocessed core antigen-envelope protein and a nonstructural protein of HCV, further preferably an NS5 nonstructural protein or a NS3-NS4 nonstructural protein. The compositions and methods may also include an inactivated preparation or an attenuated preparation comprising the proteins of the invention.

Accordingly, another aspect of the present invention provides isolated antigens capable of eliciting an immune response, preferably immunogens capable of immunizing an animal. In a preferred embodiment, the immunogens comprise amino acid sequences or molecules shown in or derived from the sequences shown in Figures 1A, 1B, 3A or 3B or substantial equivalents thereof. As will be understood by one of ordinary skill in the art, with respect to the polypeptides of the present invention, slight deviations of the amino acid sequences can be made without affecting the immunogenicity of the immunogen. Substantial equivalents of the above proteins include conservative substitutions of amino acids that maintain substantially the same charge and hydrophobicity as the original amino acid. Conservative substitutions include replacement of valine for isoleucine or leucine, and aspartic acid for glutamic acid, as well as other substitutions of a similar nature *(See* Dayhoff et al. (ed.), "Atlas of Protein Sequence and Structure," *Natl. Biomed. Res. Fdn.*, 1978).

As will be evident to one of ordinary skill in the art, the immunogens listed above, including their substantial equivalents, may stimulate different levels of response in different animals. The immunogens listed above, including their substantial equivalents, can be tested for effectiveness as a vaccine. These tests include T-cell proliferation assays, determination of lymphokine production after stimulation, and immunoprotection trials. Briefly, T-cell proliferation assays can be utilized as an indicator of potential for cell-mediated immunity. Additionally, evidence of lymphokine production after stimulation by an immunogen can be utilized to determine the potential for protection provided by an immunogen.

Finally, as described below, actual immunoprotection trials can be performed in order to determine protection in animals. In the case of humans, however,

instead of immunoprotection trials it is preferred to first screen peripheral blood lymphocytes (PBLs) from patients infected with HCV in the following manner. Briefly, PBLs can be isolated from diluted whole blood using Ficoll density gradient centrifugation and utilized in cell proliferation studies with [^{3}H]-thymidine as described below. Positive peptides are then selected and utilized in primate trials.

The immunogens, or polypeptides, of the present invention can be readily produced utilizing many other techniques well known in the art (*see* Sambrook et al., *supra, Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989).

The immunogens comprising an unprocessed core antigen-envelope protein or nonstructural protein of HCV (or both) in combination with a pharmaceutically acceptable carrier or diluent can be administered to a patient in accordance with a number procedures known in the art.

For purposes of the present invention, warm-blooded animals include, among others, humans and primates.

Many suitable carriers or diluents can be utilized in the present invention, including among others saline, buffered saline, and saline mixed with nonspecific serum albumin. The pharmaceutical composition may also contain other excipient ingredients, including adjuvants, buffers, antioxidants, carbohydrates such as glucose, sucrose, or dextrins, and chelating agents such as EDTA. Within a particularly preferred embodiment, an adjuvant is utilized along with the immunogen. Examples of such adjuvants include alum or aluminum hydroxide for humans.

The amount and frequency of administration can be determined in clinical trials, and may depend upon such factors as the HCV species against which it is desired to protect, the particular antigen used, the degree of protection required, and many other factors. In a preferred embodiment, immunizations will involve oral administration. Alternatively, the vaccine can be parenterally administrated via the subcutaneous route, or via other routes. Depending upon the application, quantities of injected immunogen will vary from 50 μg to several milligrams in an adjuvant vehicle and preferably about 100 μg to 1 mg, in combination with a physiologically acceptable carrier or diluent. Booster immunizations can be given from 4-6 weeks later.

The present invention also includes the administration of a nucleic acid vector capable of expressing the unprocessed core antigen-envelope protein or nonstructural protein of HCV (or both) into an animal, wherein the nucleic acid molecule can elicit an immune response in, and preferably immunize, an animal against the expressed protein expressed from the nucleic molecule, and therefore HCV. In one embodiment of this procedure, naked DNA is introduced into an appropriate cell, such

as a muscle cell, where it produces protein that is then displayed on the surface of the cell, thereby eliciting a response from host cytotoxic T-lymphocytes (CTLs). This can provide an advantage over traditional immunogens wherein the elicited response comprises specific antibodies. Specific antibodies are generally strain-specific and cannot recognize the corresponding antigen on a different strain. CTLs, on the other hand, are specific for conserved antigens and can respond to different strains expressing a corresponding antigen (Ulmer et al., "Heterologous protection against influenza by injection of DNA encoding a viral protein," *Science 259*:1745-1749, 1993; Lin et al., "Expression of recombinant genes in myocardium *in vivo* after direct injection of DNA," *Circulation 82*:2217-21, 1990); Wolff et al., "Long-term persistence of plasma DNA and foreign gene expression in mouse muscle," *Human Mol. Gen. 1*:363-69, 1992).

Upon introduction of the naked vector construct into the animal's cell, the construct is then able to express the nucleic acid molecule (typically a gene) that it carries, which gene preferably comprises one (or more) of the unprocessed core antigen-envelope protein or nonstructural protein of HCV. Accordingly, upon expression of the desired peptide, an immune response is elicited from the host animal. Preferably, the immune response includes $CD8^+$ CTLs able to respond to different strains that exhibit a form of the desired peptide.

Kits For The Implementation Of The Various Aspects Of The Claimed Invention

The present invention further provides kits for analyzing samples for the presence of HCV antigens or antibodies. The kits comprise a polypeptide or antibody of the invention and an appropriate solid phase. Preferably, the polypeptide is bound to the solid phase. The kits can also provide one or more reagents and/or devices for the detection of the polypeptides or antibodies. A variety of formats, reagents and devices for inclusion within the kits, including means for detecting the antigens or antibodies, are discussed herein.

The present invention also provides kits for the induction of an immune response. The kits comprise compositions comprising a polypeptide of the invention in combination with an pharmaceutically acceptable carrier or diluent, and can also provide devices for administering or assisting in the administration of the composition.

Other kits suitable for use with the features of the present invention are also provided herewith.

The following Examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

The following examples are separated into four groupings. First, Examples relating to the isolation and production of the HCV unprocessed core antigen-envelope fusion protein, and uses thereof without a nonstructural protein are presented. Second, Examples relating to the isolation and production of a nonstructural protein, and uses thereof without the HCV core antigen-envelope fusion protein are presented. Third, Examples relating to the combination and use of a nonstructural protein with the HCV core antigen-envelope fusion protein are presented. Fourth, Examples relating to the use of an HCV unprocessed core antigen-envelope fusion protein to induce an immune response in an animal are presented.

THE ISOLATION AND PRODUCTION OF AN UNPROCESSED CORE ANTIGEN-ENVELOPE PROTEIN

1. Cloning of an HCV cDNA

The plasma of patients infected with the Hepatitis C virus was collected and ultracentrifuged at 4°C and then the viral particles were obtained. Viral nucleic acid (RNA) was then extracted and purified from the viral particles using guanidine isothiocyanate and acidic phenol (Chomczynski et al., *Anal. Biochem. 162*:156-159, 1987).

The following oligonucleotide sequences:

(i) 5'-GGATCCATGAGCACAAATCCTAAACCT-3' SEQ ID No. 1)

and

(ii) 5'-GAATTCGGTGTGCATGATCATGTCCGC-3' (SEQ ID No. 2)

were used as primers in the cloning of cDNA. A single-stranded DNA molecule was produced using random primers, reverse transcriptase and the RNA template. A double-stranded DNA molecule containing the HCV core-envelope region sequence was amplified by the PCR method using Taq polymerase and primers (i) and (ii).

The cloned DNA molecule was subjected to sequence analysis for identification. The obtained molecule was designated EN-80-2. The DNA sequence of the molecule EN-80-2 is given in Fig. 1A (SEQ ID No. 7). The DNA molecule was derived from the HCV core and envelope regions and has 669bp.

2. Construction of a Plasmid Containing an HCV cDNA

The molecule EN-80-2 was treated with restriction endonucleases Bam HI and EcoRI to obtain a DNA fragment containing the desired HCV cDNA. The obtained DNA fragment was inserted into a vehicle plasmid which had been first cleaved with the restriction endonucleases Bam HI and EcoRI, to obtain an expression plasmid, designated pEN-2. The expression of the HCV cDNA is under the control of a T7 promoter. The structure of the expression plasmid pEN-2 and a restriction map are depicted in Fig. 2.

3. Transformation of *E. coli*

The expression plasmid pEN-2 was transformed into *E. coli* BL21 (DE3), spread onto an ampicillin-agar plate and placed at 37°C overnight. *E. coli* colonies producing HCV core antigen protein were selected by screening their expression products by SDS-PAGE and Western Blotting.

4. Production Of The Unprocessed Core Antigen-Envelope Protein

The transformed *E. coli* colonies were incubated in a conditioned culture medium. The colonies were centrifuged and lysed by freezing-thawing cycles and lysozyme-digestion. The unprocessed core antigen-envelope protein product was released by the lysed cells and purified by column chromatography. The polypeptide was more than 90% pure.

The unprocessed core antigen-envelope protein has a molecular weight of about 25,000 daltons as measured by electrophoresis through a sodium dodecyl sulfate-polyacrylamide gel.

5. Immunological Reactivity of HCV Core Antigen With HCV Antibodies by Western Blotting

The purified unprocessed core antigen-envelope protein was subjected to an SDS-PAGE electrophoresis using standard procedures. The SDS-PAGE gel was washed with deionized water at 4°C for 15 minutes and washed with Blotting Buffer (0.15M sodium phosphate buffer, pH 6.7) at 4°C for 20 minutes. The polypeptide on the gel was then electroblotted onto nitrocellulose membrane under the Blotting Buffer at 1.3A for 1-1.5 hours. The membrane was washed with Wash Buffer (PBS-Tween 20, pH 7.4) and blocked with Blocking Buffer (0.1M NaCl, 5mM EDTA, 50mM Tris, pH 7.2-7.4, 0.2% bovine serum albumin, 0.05% Nonidet p-40, 1M urea) overnight.

The membrane was reacted with the sera of the persons infected with/without hepatitis C, which were first diluted with 40% new born bovine

serum/Tris-HCl (pH 7.4), 10X, at 40°C for 2 hours. After the reaction, the membrane was washed with Wash Buffer three times. The membrane was reacted with an anti-hIgG:HRPO conjugate (which was prepared as described hereafter) at 40°C for 2 hours. After the reaction, the membrane was washed with Wash Buffer three times and then reacted with 10 ml Substrate Solution.(0.01% 4-chloro-1-Naphthol, 18% methanol, 0.04M Tris, pH 7.2-7.4, 0.1 M NaCl and 0.01% H_2O_2) for 20 minutes. The unprocessed core antigen-envelope protein of the present invention was reactive with the sera of HCV patients but not reactive with the sera of healthy persons.

6. ELISA for HCV Antibodies

(A) Treatment of Microtiter Plate

A microtiter plate was coated with the purified unprocessed core antigen-envelope protein of the invention at appropriate concentrations and blocked with a buffer containing bovine serum albumin. The treated microtiter plate was stored at 2-8°C.

(B) Preparation of Anti-hIgG:HRPO Conjugate

Purified anti-human Immunoglobulin G (anti-hIgG) was conjugated with horse radish peroxidase (HRPO) using $NaIO_4$ to obtain the anti-IgG:HRPO conjugate. The conjugate was purified by chromatography.

(C) Components of Reagents

(a) Wash Solution: Phosphate Buffer containing 0.9% NaCl and Thimerosal.

(b) Anti-hIgG:HRPO Conjugate Solution: the anti-hIgG:HRPO conjugate prepared as described above dissolved in Tris Buffer containing a proteineous stabilizer and antiseptics.

(c) Sample Diluent: Tris Buffer containing a proteineous stabilizer and antiseptics.

(d) OPD Substrate Solution: o-phenylene diamine (OPD) dissolved in citrate-phosphate buffer containing H_2O_2. (If the solution becomes orange, it means that the solution has been contaminated and cannot be used any more.)

(e) Stopping Solution: 2N H_2SO_4 solution.

(f) Positive/Negative controls: the serum samples of persons infected with/without hepatitis C diluted with phosphate buffer containing

a proteineous stabilizer and antiseptics at an appropriate concentration.

(D) Procedure:

(a) One hundred and fifty microliters (μl) of the test samples were diluted with Sample Diluent (1:10), and Positive/Negative Controls were added into the wells of the treated microtiter plate. Some wells were retained as substrate blanks.

(b) The plate was gently mixed by shaking and incubated at 37-40°C for 1 hour.

(c) The plate was washed three times with 0.3 ml of Wash solution per well.

(d) One hundred μl of anti-hIgG:HRPO Conjugate Solution was added to each well.

(e) The plate was gently mixed by shaking and incubated at 37-40°C for 30 minutes.

(f) The plate was washed five times.

(g) One hundred μl of OPD Substrate Solution was added to each well and the plate was incubated at 15-30°C in the dark for 30 minutes.

(h) One hundred μl of Stopping Solution was added to each well and gently mixed to stop the reaction.

(i) The OD value per well was measured at 492 nm in a spectrophotometer.

(E) Determination:

The OD_{492nm} value per well subtracts the mean of the readings of the blanks (backgrounds). The difference (PCx-NCx) between the mean of the readings of the positive controls (PCx) and that of the negative controls (NCx) is equal to or more than 0.5.

The Cut-off value (CO) is calculated by the following formula:

$$CO = PCx \times 0.15 + NCx$$

When the readings from test samples were less than the CO value, the samples were considered negative (*i.e.*, HCV antibodies could not be detected in the samples).

When the readings of test samples were equal to or more than the CO value, the samples were expected to be positive; however, it is preferred to repeat the assay for the samples in duplicate. If the readings of either of the duplicate samples were less than the CO value, the samples were considered to be negative. If the duplicate samples were both more than or equal to the Cut-off value, the samples were considered to be positive.

When the readings of test samples are more than NCx but less than the CO value by 20%, the samples should be regarded as questionable samples and the assay has to be repeated for those samples.

Twenty-seven samples were tested by the ELISA according to the invention. At the same time, the samples were also tested with the Abbott's kit (II) HCV antibody assay, which kit contains both structural and nonstructural proteins (*i.e.*, core (amino acids 1-150), NS-3 and NS-4). The comparison between the test results of Abbott's kit (II) and those of the assay of the present invention is given in Table 1. It is noted that the results of Sample G 229 were negative according to Abbott's kit (II), but were positive according to the assay of the present invention. Sample G 229 was confirmed to be positive for HCV.

TABLE 1

Sample No.		OD_{492nm}	Results	References Abbott's kit (II)
TSGH	56	> 2.0	positive	positive
TSGH	57	> 2.0	positive	positive
G	23	1.469	positive	positive
G	30	> 2.0	positive	positive
G	32	> 2.0	positive	positive
G	49	> 2.0	positive	positive
G	56	> 2.0	positive	positive
G	58	> 2.0	positive	positive
G	114	1.559	positive	positive
G	128	> 2.0	positive	positive
G	186	> 2.0	positive	positive
G	208	> 2.0	positive	positive

G	214	> 2.0	positive	positive
G	231	> 2.0	positive	positive
G	250	> 2.0	positive	positive
Y	1	> 2.0	positive	positive
USB	9	> 2.0	positive	positive
USB	19	> 2.0	positive	positive
USB	20	> 2.0	positive	positive
USB	23	0.952	positive	positive
USB	27	0.753	positive	positive
G	11	0.147	negative	negative
G	12	0.077	negative	negative
G	13	0.061	negative	negative
G	14	0.116	negative	negative
G	15	0.139	negative	negative
G	229	0.517	**positive**	**negative**

THE ISOLATION AND PRODUCTION OF HCV NONSTRUCTURAL PROTEINS

7. Cloning of an HCV cDNA Encoding The NS5 Nonstructural Protein

The plasma of patients infected with Hepatitis C virus was collected and ultracentrifuged at 4°C and then the viral particles were obtained. Subsequently, the viral nucleic acid (RNA) was extracted and purified from the viral particles using guanidine isothiocyanate and acidic phenol (Chomczynski et al., *Anal. Biochem.* *162*:156-159, 1987).

The following oligonucleotide sequences:

(i) 5'-GGATCCCGGTGGAGGATGAGAGGGAAATATCCG-3'
(SEQ ID No. 3)
and
(ii) 5'-GAATTCCCGGACGTCCTTCGCCCCGTAGCCAAATTT-3'
(SEQ ID No. 4)

were used as primers in the cloning of cDNA. A single-stranded DNA molecule was produced using random primers, reverse transcriptase and the RNA template. A double-

stranded DNA molecule containing the HCV NS5 sequence was amplified by the PCR method using Taq polymerase and primers (i) and (ii).

The cloned DNA molecule was subjected to sequence analysis for identification. The obtained molecule was designated EN-80-1. The DNA sequence of the molecule EN-80-1 is given in Figure 3A, and the amino acid sequence encoded by the molecule is given in Figure 3B. The DNA molecule was derived from the genome of HCV nonstructural region 5 and has 803 bp (SEQ ID No. 9). The amino acid sequence of the molecule EN-80-1 is given in Fig. 3B (SEQ ID No. 10), and has 267 residues.

8. Construction of a Plasmid Containing an HCV cDNA

The molecule EN-80-1 was treated with restriction endonucleases Bam HI and EcoRI to obtain a DNA fragment containing said HCV cDNA. The resulting DNA fragment was inserted into a vehicle plasmid which had been first cleaved with restriction endonucleases Bam HI and EcoRI, to obtain an expression plasmid, designated pEN-1. The expression of the HCV cDNA is under the control of a T7 promoter. The structure of the expression plasmid pEN-1 and restriction map are given in Fig. 4.

9. Transformation of *E. coli*

The expression plasmid pEN-1 was transformed into *E. coli* BL21 (DE3), spread onto an ampicillin-agar plate and placed at 37°C overnight. *E. coli* colonies producing the HCV nonstructural protein were selected by screening their expression products by SDS-PAGE and Western Blotting.

10. Production of The NS5 Nonstructural Protein

The transformed *E. coli* colonies were incubated in a conditioned culture medium. The colonies were centrifuged and lysed by freezing-thawing cycles and lysozyme-digestion. The protein product was released by the lysed cells and purified by column chromatography. The resulting polypeptide was more than 90% pure.

The polypeptide has a molecular weight of about 29,000 daltons as measured by electrophoresis through a sodium dodecyl sulfate-polyacrylamide gel.

11. Immunological Reactivity of The NS5 Nonstructural Protein With HCV Antibodies by Western Blotting

The purified polypeptide was subjected to SDS-polyacrylamide gel electrophoresis using standard procedures. The SDS-PAGE gel was washed with

deionized water at 4°C for 15 minutes and washed with Blotting Buffer (0.15M sodium phosphate buffer, pH 6.7) at 4°C for 20 minutes. The polypeptide on the gel was then electroblotted onto a nitrocellulose paper under the Blotting Buffer at 1.3A for 1-1.5 hours. The membrane was washed with Wash Buffer (PBS-Tween 20, pH 7.4) and blocked with Blocking Buffer (0.1M NaCl, 5mM EDTA, 50mM Tris, pH 7.2-7.4, 0.2% bovine serum albumin, 0.05% Nonidet p-40, 1M urea) overnight.

The membrane was reacted with the sera of the persons infected with/without hepatitis C, which were first diluted with 40% New Born Bovine Serum/Tris-HCl (pH 7.4), 10X, at 40°C for 2 hours. After the reaction, the membrane was washed with Wash Buffer three times. The membrane was then reacted with an anti-hIgG:HRPO conjugate (which is prepared as described hereafter) at 40°C for 2 hours. After the reaction, the membrane was washed with Wash Buffer three times and then reacted with 10 ml Substrate Solution (0.01% 4-chloro-1-Naphthol, 18% methanol, 0.04M Tris, pH 7.2-7.4, 0.1 M NaCl and 0.01% H_2O_2) for 20 minutes. The polypeptide of the present invention was reactive with the sera of HCV patients but was not reactive with the sera of healthy persons.

12. ELISA for HCV Antibodies

(A) Treatment of Microtiter Plate

A microtiter plate was coated with the NS5 nonstructural protein of the invention at appropriate concentrations and blocked with a buffer containing bovine serum albumin. The treated microtiter plate was stored at 2-8°C.

(B) Preparation of Anti-hIgG:HRPO Conjugate

The purified anti-human Immunoglobulin G (anti-hIgG) was conjugated with horse radish peroxidase (HRPO) using $NaIO_4$ to obtain the anti-IgG:HRPO conjugate. The conjugate was purified by chromatography.

(C) Components of Reagents

(a) Wash Solution: Phosphate Buffer containing 0.9% NaCl and Thimerosal.

(b) Anti-hIgG:HRPO Conjugate Solution: the anti-hIgG:HRPO conjugate prepared as described above dissolved in Tris Buffer containing a proteineous stabilizer and antiseptics.

(c) Sample Diluent: Tris Buffer containing a proteineous stabilizer and antiseptics.

(d) OPD Substrate Solution: o-phenylene diamine (OPD) dissolved in citrate-phosphate buffer containing H_2O_2. (If the solution becomes orange, it means that the solution has been contaminated and cannot be used any more.)

(e) Stopping Solution: 2N H_2SO_4 solution.

(f) Positive/Negative controls: the serum samples of persons infected with/without hepatitis C diluted with phosphate buffer containing a proteineous stabilizer and antiseptics at an appropriate concentration.

(D) Procedure:

(a) One hundred and fifty microliters (µl) of test samples diluted with Sample Diluent (1:10) and Positive/Negative Controls were added to the wells of the treated microtiter plate. Some wells were retained as substrate blanks.

(b) The plate was gently mixed by shaking and incubated at 37-40°C for 1 hour.

(c) The plate was washed three times with 0.3 µl of Wash Solution per well.

(d) One hundred µl of anti-hIgG:HRPO Conjugate Solution was added to each well.

(e) The plate was gently mixed and incubated by shaking at 37-40°C for 30 minutes.

(f) The plate was washed five times.

(g) One hundred µl of OPD Substrate Solution was added to each well and the plate was incubated at 15-30°C in the dark for 30 minutes.

(h) One hundred µl of Stopping Solution was added to each well and gently mixed to stop the reaction.

(i) The OD value per well was measured at 492 nm in a spectrophotometer.

(E) Determination:

The OD_{492nm} value per well subtracts the mean of the readings of the blanks (backgrounds). The difference (PCx-NCx) between the mean of the readings of

the positive controls (PCx) and that of the negative controls (NCx) is equal to or more than 0.5.

The Cut-off value (CO) was calculated by the following formula:

$$CO = PCx \times 0.15 + NCx$$

When the readings of test samples were less than the CO value, the samples were considered to be negative (*i.e.*, HCV antibodies could not be detected in the samples). When the readings of test samples were equal to or more than the CO value, the samples were expected to be positive; however, it is preferred to repeat the assay for the samples in duplicate. If the readings of either of the duplicate samples were less than the CO value, the samples will be negative. If the duplicate samples were both more than or equal to the CO value, the samples were considered to be positive.

When the readings of the test samples are more than NCx but less than the CO value by 20%, the samples should be regarded as questionable samples and the assay has to be repeated for the samples.

Eighteen samples were tested by the ELISA according to the invention. At the same time, the samples were also tested with the Abbott's kit (I) HCV antibody assay, which kit contains nonstructural protein C100-3, and with the Abbott's kit (II) HCV antibody assay, which kit contains both structural and nonstructural proteins The comparison between the test results of the Abbott's kits and those-of the assay of the invention is given in Table 2. It is noted that the results of Sample G 30 and Sample G 128 were negative according to Abbott's kit (I) but were positive according to the assay of the present invention. These samples were confirmed to be positive for HCV.

TABLE 2

Sample No.		OD_{492nm}	Results	References Abbott's kit	
				(I)	(II)
TSGH	56	> 2.0	positive	positive	positive
G	23	0.813	positive	positive	positive
G	26	1.607	positive	positive	positive
G	30	> 2.0	**positive**	**negative**	**positive**
G	32	> 2.0	positive	positive	positive
G	56	> 2.0	positive	positive	positive
G	128	> 2.0	**positive**	**negative**	**positive**

G	186	> 2.0	positive	positive	positive
G	208	> 2.0	positive	--	positive
G	214	> 2.0	positive	--	positive
G	231	> 2.0	positive	--	positive
Y	1	> 2.0	positive	--	positive
USB	9	> 2.0	positive	--	positive
USB	19	> 2.0	positive	--	positive
USB	20	> 2.0	positive	--	positive
G	201	0.062	negative	--	negative
G	202	0.072	negative	--	negative
G	211	0.059	negative	--	negative

DETECTION USING BOTH UNPROCESSED CORE ANTIGEN-ENVELOPE PROTEIN AND A NONSTRUCTURAL PROTEIN

13. ELISAs For HCV Using Both Unprocessed Core Antigen-Envelope Protein And An NS5 Nonstructural Protein

A. ASSAYS COMPARING THE CORE ANTIGEN-ENVELOPE PROTEIN AND THE NS5 NONSTRUCTURAL PROTEIN WITH ABBOTT'S HCV ASSAYS

1. FIRST ASSAY

The method was analogous to the ELISAs described above, except that unprocessed core antigen-envelope protein was combined with an NS5 nonstructural protein (9:1) (known as the EverNew Anti-HCV EIA).

In a first assay, twenty-four samples were tested by the above-described method. At the same time, the samples were also tested by Abbott's kit (II). The results are given in Table 3. In this assay, the results of the Abbott's kit (II) were the same as the assay using the antigens of the present invention.

TABLE 3

Sample No.		OD_{492nm}	Results	References Abbott's kit (II)
TSGH	56	> 2.0	positive	positive
TSGH	57	> 2.0	positive	positive
G	23	1.469	positive	positive
G	26	> 2.0	positive	positive
G	30	> 2.0	positive	positive
G	32	> 2.0	positive	positive
G	49	> 2.0	positive	positive
G	56	> 2.0	positive	positive
G	58	> 2.0	positive	positive
G	114	> 2.0	positive	positive
G	128	> 2.0	positive	positive
G	186	> 2.0	positive	positive
G	214	> 2.0	positive	positive
G	231	> 2.0	positive	positive
G	250	> 2.0	positive	positive
Y	1	> 2.0	positive	positive
USB	9	> 2.0	positive	positive
USB	19	> 2.0	positive	positive
USB	20	> 2.0	positive	positive
USB	23	> 2.0	positive	positive
USB	27	> 2.0	positive	positive
G	92	0.038	negative	negative
G	93	0.056	negative	negative
G	94	0.071	negative	negative

II. SECOND ASSAY

The clinical trial report of blood donors for EverNew Anti-HCV EIA is shown in TABLE 4:

Hospital: Taipei Tri-Service General Hospital

Sample Source: Collected from Blood Bank

Classification of Sample: Volunteer Blood Donors

Reference Kit: Abbott's Kit (II)

Results:

TABLE 4

		ABBOTT +	ABBOTT -	Total
EverNew	+	5 (2.5%)	1 (0.5%)	6 (3%)
	-	1 (0.5%)	193 (96.5%)	194 (97%)
	total	6 (3%)	194 (97%)	200 (100%)

The results in Table 4 indicate that both assays provided the same detection.

III. THIRD ASSAY

The clinical trial report of high risk patients for EverNew Anti-HCV EIA is shown in TABLE 5:

Hospital: Taipei Veteran General Hospital

Sample Source: Collected from Department of Clinical Virology

Classification:

NANB, sporadic	20
NANB, PHT	12
HCC	15
Liver cirrhosis	9
Chronic hepatitis B and carrier	10
Biliary tract stones	4
Alcoholic liver disease	3
Fatty liver	2
Acute hepatitis, etiology?	2
Schistosomiasis of liver	1
Hepatic cysts	1
Cholangio-CA	1
Non-hepatobiliary disease	6
No data	2
Total	88

Reference Kit: Abbott's kit (II)

Results:

TABLE 5

		ABBOTT +	ABBOTT -	Total
EverNew	+	54 (61.36%)	0 (0%)	54 (61.36%)
	-	1 (1.14%)@	33 (37.5%)	34 (38.64%)
	total	55 (62.5%)	33 (37.5%)	88 (100%)

@: HCV RT/PCR Method: Negative

The clinical data and the HCV RT/PCR results indicate that the efficiency of the EverNew Anti-HCV EIA for HCV antibody detection was better than the Abbott's kit (II) licensed by the U.S. FDA.

B. ASSAYS SHOWING THE SYNERGISTIC COOPERATION OF THE CORE ANTIGEN-ENVELOPE PROTEIN AND A SECOND PROTEIN

I. FIRST ASSAY

This assay shows the results of an ELISA similar to those set forth above, and shows cooperative interaction between EN-80-2 and EN-80-1 proteins of HCV. The protocol for the ELISA is as follows:

Coating buffer: 0.05 M Tris-HCl/ 0.15 N NaCl/6 M Urea pH: 7.4 ± 0.2.

Washing buffer: PBS with 0.05% Tween 20.

Postcoating buffer: PBS buffer with 1% BSA.

Coating procedure: EN-80-1 and EN-80-2 proteins were added into coating buffer (final concentration: about 1.5 µg/ml) and mixed at room temperature for 30 minutes. After mixing, the diluted EN-80-1 and EN-80-2 proteins were added into microtiter wells, 100 µg/well, and incubated in a 40°C incubator for 24 hours. The microtiter wells were then washed, and postcoating buffer was added into the wells. The microtiter wells were then let stand at 4°C for overnight. After postcoating, the coated microtiter wells can be used for anti-HCV antibody detection.

Sample diluent: 0.1 M Tris-HCl pH: 7.4 ± 0.2 with 40% NBBS, 1% BSA and 2% mouse serum.

Conjugate: anti-human IgG monoclonal antibody coupled with HRPO using $NaIO_4$. After coupling, the anti-human IgG:HRPO conjugates were purified by S-200 gel filtration and were diluted in sample diluent.

OPD tablets: purchased from Beckman.

Substrate diluent: citrate-phosphate buffer containing H_2O_2.

Stopping solution: 2N H_2SO_4.

Positive control: anti-HCV positive serum diluted in sample diluent.

Negative control: recalcified human serum, which is non-reactive for HBV markers, anti-HIV, anti-HTLV I and anti-HCV.

Assay procedure:

100 µl sample diluent was added into each well.

50 µl sample, positive control and negative control was added into appropriate wells.

Sample incubation: incubated at 40 ± 1°C for 30 ± 2 minutes.

Sample wash: the wells were washed 3 times using washing buffer.

100 µl anti-human IgG:HRPO conjugate was added into each well.

Conjugate incubation: incubated at 40 ± 1°C for 30 ± 2 minutes.

Conjugate wash: the wells were washed 6 times using washing buffer.

After washing, 100 µl substrate solution was added (the substrate solution was prepared by dissolving one tablet OPD in 5 ml substrate diluent), then the mixture was allowed to stand at room temperature for 10 minutes. In order to prevent light, the microtiter wells were covered with a black cover.

100 µl stopping solution was added into each well. Gently mix.

Evaluation: The OD value per well was measured at 492 nm in a spectrophotometer.

Interpretation:

Determination of cutoff value: cutoff value = PCx X 0.25 + NCx.

An absorbance equal to or greater than cutoff value indicated that a reaction was considered to be positive, which means reactive for anti-HCV antibody. An absorbance less than the cutoff value was considered to be negative, which means non-reactive for anti-HCV antibody.

The sample sources for the assay reflected in Table 6 were as follows:

Sample source I: G83, G191, G205 and G235 were GPT abnormal samples that were anti-HCV antibody negative and were collected from Taipei blood donation center.

Sample source II: G614 and G615 were anti-HCV antibody positive and were purchased from the U.S.A.

Sample source III: 8-5 was anti-HCV antibody positive and was collected from the Taichung blood donation center.

Sample source IV: N345 was a patient serum.

TABLE 6

Sample		EN-80-1	EN-80-2	EN-80-1 + EN-80-2
G83		0.027@	0.047	0.055
G191		0.071	0.209	0.056
G205		0.027	0.034	0.039
G235		0.025	0.044	0.043
G614	8X#	0.066	0.831	1.894
G614	16X	0.059	0.348	0.848
G615	8X	0.048	0.495	1.592
G615	16X	0.053	0.209	0.740
8-5		0.059	0.352	0.690
N345$		0.008	0.420	0.730

@: Absorbance at 492 nm.

#: Samples were diluted with recalcified human serum, which is non-reactive for HBV, HCV and HIV.

$: Abbott's kit (II) found this sample to be negative.

These data demonstrate that when the EN-80-2 and EN-80-1 proteins were combined, the absorbance at 492 nm for anti-HCV positive samples was synergistic, not additive. Thus, cooperative interactions between EN-80-2 and EN-80-1 proteins of HCV were found. One benefit of this synergism is shown, for example, with sample N345, which was found to be HCV negative by Abbott's kit (II), but due to the synergistic effect was found to be positive by the present invention. These data also demonstrate that the synergistic effect is helpful in screening for anti-HCV antibodies in samples, particularly in early detection situations.

II. SECOND ASSAY

This assay was conducted as set forth in the First Assay, above, and included the provision in a single well of a core-envelope fusion protein of the invention in combination with an NS3-NS4 protein identified as EN-80-4. The results of the ELISA are set forth in Table 7.

TABLE 7

Sample		EN-80-2	EN-80-4	EN-80-2 + EN-80-4
G83		0.047@	0.032	0.049
G191		0.209	0.103	0.102
G205		0.034	0.045	0.046
G235		0.044	0.064	0.068
G58	21X#	0.561	0.041	1.729
G612	161X	1.298	0.218	>2.0
G613	40X	0.202	0.243	0.708

@: Absorbance at 492 nm.

#: Samples were diluted with recalcified human serum, which is non-reactive for HBV, HCV and HIV.

The data in Table 7 demonstrate that when the EN-80-2 and EN-80-4 proteins were combined, the absorbance at 492 nm for anti-HCV positive samples showed a synergistic effect, not merely an additive effect. Thus, cooperative interactions between EN-80-2 and EN-80-4 proteins of HCV were found.

III. THIRD ASSAY

This assay shows the results of an ELISA performed according to the protocol set forth in the First Assay, above, wherein a partial core protein was combined with the EN-80-1 (NS5) protein of HCV. The partial core protein consisted of amino acids 1 through 120, and was a gift from the Development Center of Biotechnology (DCB) in Taiwan.

Sample source I: G235 was a GPT abnormal sample, which was anti-HCV antibody negative and was collected from the Taipei blood donation center.

Sample source II: G614 and G615 were anti-HCV positive samples and were purchased from the U.S.A.

TABLE 8

Sample		EN-80-1	partial core	EN-80-1 + partial core
G235		0.002@	0.082	0.078
G614		0.004	1.142	1.243
G615		0.000	1.332	1.430

@: Absorbance at 492 nm.

The data in Table 8 demonstrate that when the partial core (amino acids 1 through 120) and EN-80-1 proteins were coated together, the absorbance at 492 nm of anti-HCV positive samples was not synergistic. No cooperative interaction between partial core and NS5 proteins of HCV were found.

IV. FOURTH ASSAY

Table 9 depicts the results of an assay that was similar to that in the Fifth Assay (V), and shows that there were no cooperative interactions between the HBV antigens HBsAg and HBcAg and the EN-80-1 protein of HCV.

HBsAg: purified from HBsAg positive human plasma.

HBcAg: derived from HBV cDNA fragment.

Sample source I: G30 and G49 were GPT abnormal samples, which were anti-HCV antibody positive and were collected from the Taipei Blood Donation Center.

Sample source II: G612, G613, G614 and G615 were anti-HCV antibody positive and were purchased from the United States of America.

TABLE 9

Sample.		EN-80-1	HBsAg	HBcAg	EN-80-1 + HBsAg	EN-80-1 + HBcAg
G30	102X@	0.088#	0.117	0.162	0.186	0.219
G49	42X	0.063	0.125	0.174	0.146	0.190
G612	804X	0.096	0.111	0.145	0.178	0.187
G613	52X	0.195	0.165	0.137	0.232	0.239
G614	16X	0.059	0.124	0.123	0.111	0.116
G615	16X	0.053	0.107	0.134	0.158	0.232

@: Samples were serially diluted with recalcified human serum, which was non-reactive for HBV, HCV, and HIV.

#: Absorbance at 492 nm.

The data in Table 9 demonstrate that when the HBsAg or the HBcAg were coated together with the EN-80-1 (NS5) protein, the absorbance of anti-HCV positive samples was not synergistic. No apparent interactions between the HBsAg and the EN-80-1 protein, or the HBcAg and the EN-80-1 protein, were found.

V. FIFTH ASSAY

Table 10 shows a comparison of the detection of anti-HCV antibodies between the EverNew Anti-HCV EIA and the Abbott's kit (II). The samples for the test were obtained from the following sources:

Sample source I: G23, G26, G30, G32, G49, G58, G114, G128, G186, G231, G250 and G262 were GPT abnormal samples, which were anti-HCV antibody positive and were collected from Taipei blood donation center.

Sample source II: G612, G613, G614 and G615 were anti-HCV antibody positive and were purchased from U.S.A.

Sample source III: VGH7, VGH11, VGH12, VGH13, VGH16, VGH26, VGH27, VGH29, VGH30, VGH32, VGH33, VGH40, VGH43, VGH46 and VGH52 were anti-HCV antibody positive and were collected from Taipei Veteran General Hospital.

Classification for the samples from source III:

VGH7	IHD stones
VGH11	NANB, sporadic
VGH12	NANB, sporadic
VGH13	NANB, PTH
VGH16	HCC
VGH26	Liver cirrhosis
VGH27	NANB, sporadic
VGH29	IHD stone
VGH30	Schistosomiasis of liver
VGH32	NANB, sporadic
VGH33	Liver cirrhosis
VGH40	No data
VGH43	NANB, sporadic
VGH46	Liver cirrhosis with HCC
VGH52	NANB, sporadic

Control: Recalcified human serum (non-reactive with HBV, anti-HCV and HIV). This human serum was also used to dilute the above-mentioned anti-HCV positive samples.

Tested Kits:

EverNew Anti-HCV EIA --- Microtiter wells coated with EN-80-1 antigen.

EverNew Anti-HCV EIA --- Microtiter wells coated with EN-80-2 antigen.

EverNew Anti-HCV EIA --- Microtiter wells coated with EN-80-1 and EN-80-2 antigens.

Reference Kit: Abbott's kit (II).

Results:

TABLE 10

Sample	Dilution	EN-80-1	EN-80-2	EN-80-1 + EN-80-2	ABBOTT
Recalcified human serum (Control)	n/a	negative	negative	negative	negative
G23	20X @	negative $	positive #	positive	positive
	40X	negative	negative	positive	positive

G26	8X	negative	positive	positive	positive
	16X	negative	negative	positive	positive
G30	51X	negative	negative	positive	positive
	102X	negative	negative	positive	positive
G32	51X	positive	negative	positive	positive
	102X	negative	negative	positive	positive
G49	21X	negative	negative	positive	positive
	42X	negative	negative	positive	positive
G58	16X	negative	positive	positive	positive
	32X	negative	negative	positive	positive
G114	10X	negative	positive	positive	positive
	20X	negative	negative	positive	positive
G128	120X	negative	negative	positive	positive
	240X	negative	negative	positive	negative
G186	42X	negative	negative	positive	positive
	84X	negative	negative	negative	negative
G231	336X	negative	negative	positive	positive
	672X	negative	negative	positive	negative
G250	168X	negative	negative	positive	positive
	336X	negative	negative	positive	positive
G262	84X	negative	positive	positive	positive
	168X	negative	negative	positive	positive
G612	402X	negative	negative	positive	positive
	804X	negative	negative	positive	negative
G613	26X	negative	negative	positive	positive
	52X	negative	negative	positive	positive
G614	8X	negative	positive	positive	positive
	16X	negative	negative	positive	positive
G615	8X	negative	positive	positive	positive
	16X	negative	negative	positive	positive

VGH7	42X	negative	positive	positive	positive
	84X	negative	negative	positive	positive
VGH11	126X	positive	negative	positive	positive
	252X	negative	negative	positive	positive
VGH12	252X	negative	negative	positive	positive
	504X	negative	negative	positive	positive
VGH13	252X	negative	positive	positive	positive
	504X	negative	negative	positive	positive
VGH16	252X	negative	negative	positive	positive
	504X	negative	negative	positive	positive
VGH26	84X	negative	negative	positive	positive
	168X	negative	negative	positive	positive
VGH27	42X	negative	negative	positive	negative
	84X	negative	negative	positive	negative
VGH29	42X	negative	positive	positive	positive
	84X	negative	negative	positive	negative
VGH30	42X	positive	negative	positive	positive
	84X	negative	negative	positive	negative
VGH32	504X	negative	negative	positive	negative
	1008X	negative	negative	positive	negative
VGH33	84X	negative	positive	positive	negative
	168X	negative	negative	positive	negative
VGH40	9X	negative	negative	positive	negative
	18X	N.D. &	N.D.	negative	negative
VGH43	9X	negative	negative	positive	negative
	18X	N.D.	N.D.	positive	negative
VGH46	9X	negative	negative	positive	positive
	12X	N.D.	N.D.	positive	positive
VGH52	126X	negative	negative	positive	positive
	252X	negative	negative	negative	negative

@: Samples were serially diluted with recalcified human serum which was non-reactive with HBV, anti-HCV and HIV.

$: negative --- non-reactive with anti-HCV antibody.

#: positive --- reactive with anti-HCV antibody.

&: N.D. --- not done.

The data in Table 10 in bold show instances of synergy between the core antigen-envelope protein and the nonstructural (NS5) region of HCV. The data in bold also demonstrate instances where the invention provided better detection than the reference Abbott's kit (II) HCV detection kit. These data indicate that the detectability of the microtiter wells coated with EN-80-1 and EN-80-2 antigens was more efficient than the microtiter wells coated with either EN-80-1 antigen or EN-80-2 antigen alone. Furthermore, anti-HCV antibody in samples G128 240X, G231 672X, G612 804X, VGH27 42X, VGH27 84X, VGH29 84X, VGH30 84X, VGH32 504X, VGH32 1008X, VGH33 84X, VGH33 168X, VGH40 9X, VGH43 9X and VGH43 18X could be detected by using EverNew Anti-HCV EIA (microtiter wells coated with EN-80-1 and EN-80-2 antigens) but was not detected using the Abbott's kit (II).

VI. SIXTH ASSAY

Table 11 confirms the above-presented results and shows an enzyme immunoassay comparison of the detection of anti-HCV antibodies using partial core (EN-80-5 antigen, which is an HCV partial core antigen having a molecular weight of about 15,000 daltons as measured by electrophoresis through a SDS-polyacrylamide gel), core antigen-envelope protein (EN-80-2 antigen) and/or an HCV nonstructural protein (NS5; the EN-80-1 antigen discussed above). The samples for the assay were anti-HCV positive samples nos. N8, N81, N89, N12 and N302, and anti-HCV negative samples nos. N202, N203 and N302. The positive samples were diluted between 25X and 672X with 0.1M Tris-HCl, pH 7.4 (+/- 0.2) with 40% new born bovine serum, 1% BSA and 2% mouse serum. The samples were assayed in microtiter wells with a monoclonal anti-human IgG:HRPO conjugate solution, in combination with the following antigens or combinations of antigens: a.) NS5; b.) core antigen-envelope protein; c.) partial core protein; d.) NS5 and core antigen-envelope protein; e.) core antigen-envelope protein and partial core; and, f.) NS5, core antigen-envelope protein, and partial core.

The following results were obtained:

Table 11

Sample ID		NS5	core-env	core	NS5 + core-env	NS5 + core	core + core-env	NS5 + core + core-env
N8	50X @	0.098*	1.009	0.952	> 2.0	0.535	> 2.0	> 2.0
	100X	0.047	0.473	0.400	0.869	0.228	0.781	0.781
N81	336X	0.018	1.572	1.778	> 2.0	0.696	> 2.0	> 2.0
	672X	0.019	0.697	0.633	0.742	0.344	0.912	0.982
N89	336X	0.083	> 2.0	> 2.0	> 2.0	1.918	> 2.0	> 2.0
	672X	0.040	1.301	0.794	1.671	0.589	1.321	1.694
N12	25X	0.019	1.848	> 2.0	> 2.0	0.676	> 2.0	> 2.0
	50X	0.013	0.775	0.898	1.587	0.278	1.297	0.966
	100X	0.009	0.333	0.317	0.566	0.092	0.390	0.435
N302	168X	0.188	> 2.0	> 2.0	> 2.0	> 2.0	> 2.0	> 2.0
	336X	0.078	1.161	1.968	1.645	1.660	> 2.0	> 2.0
	672X	0.046	0.496	0.819	0.829	0.612	0.805	1.025
N202		0.043	0.081	0.069	0.077	0.048	0.081	0.075
N203		0.100	0.208	0.124	0.185	0.117	0.189	0.169
N209		0.023	0.033	0.054	0.036	0.037	0.045	0.042
Sample diluent		0.018	0.028	0.018	0.021	0.025	0.028	0.027

@: Anti-HCV positive serum diluted with sample diluent (0.1 M Tris-HCl pH: 7.4±0.2 with 40% new born bovine serum, 1% BSA and 2% mouse serum).

*: Absorbance at 492nm.

THE PRODUCTION OF MONOCLONAL ANTIBODIES TO THE UNPROCESSED CORE ANTIGEN-ENVELOPE PROTEIN

14. Preparation of Antibodies Against HCV

Antibodies against unprocessed core antigen-envelope protein and the NS5 nonstructural protein were produced according to a standard procedure for producing monoclonal antibodies. In particular, a BALB/c mouse was immunized with

the purified proteins described above in Examples 2 and 10, mixed with an adjuvant; and then the spleen cells were fused with mouse myeloma cells (FO cell line) using polyethylene glycol to form hybridoma cells. The desired clones producing desired monoclonal antibodies was obtained by screening the titer of the antibodies produced by the hybridoma clones so prepared. In one embodiment of the invention, a hybridoma clone was designated EN-80-1-99.

THE USE OF AN HCV UNPROCESSED CORE ANTIGEN-ENVELOPE PROTEIN TO INDUCE AN IMMUNE RESPONSE

15. Administration Of A Core Antigen-Envelope Protein

A core antigen-envelope protein (EN-80-2) was administered intramuscularly to ICR mice at 6-8 weeks of age. The first administration, boost and sampling schedule was as follows:

Negative control Group: (ID nos. 0-1 and 0-2)
Day 0: no immunization.
Day 13: 1st bleeding
Day 28: 2nd bleeding

Test Group 1: (ID nos. 1-1, 1-2, 1-3, 1-4, 1-5 and 1-6)
Day 0: 50 μg/mouse of EN-80-2 protein using complete Freund's adjuvant (CFA), GIBCO (Gaithersburg, MD, USA, 20877).
Day 13: 1st bleeding
Day 28: 2nd bleeding
Day 39: 3rd bleeding

Test Group 2: (ID nos. 2-1, 2-2, 2-3, 2-4, 2-5 and 2-6)
Day 0: 50 μg/mouse of EN-80-2 protein using complete Freund's adjuvant (CFA), GIBCO.
Day 13: 1st boost, with 80 μg/mouse of EN-80-2 protein using incomplete Freund's adjuvant (IFA), also from GIBCO (Gaithersburg, MD, USA, 20877).
Day 28: 1st bleeding
Day 39: 2nd bleeding

Test Group 3: (ID nos. 3-1, 3-2, 3-3, 3-4, 3-5 and 3-6)
Day 0: 50 μg/mouse of EN-80-2 protein using complete Freund's adjuvant (CFA), GIBCO.
Day 13: 1st boost, with 80 μg/mouse of EN-80-2 protein using incomplete Freund's adjuvant (IFA), GIBCO.

Day 28: 2nd boost, with 80 µg/mouse of EN-80-2 protein, in PBS.
Day 39: 1st bleeding

16. Detection Of The Immune Response Induced By The Administration Of the Core Antigen-Envelope Protein

The presence or absence of an immune response in the test animals was determined using two enzyme immunoassays (EIAs) similar to those described above. In the first EIA, a rat anti-mouse:HRPO conjugate was added to the wells of a microtiter plate that had been coated a core antigen-envelope protein (EN-80-2) along with a rat anti-mouse:HRPO conjugate. The results of the first EIA are shown below in Table 12.

Table 12

Sample ID		Day 13	Day 28	Day 39
Negative control:				
0-1	50X @	0.141 #	0.160	N.D. $
	500X	0.058	0.060	N.D.
	2500X	0.008	0.025	N.D.
	12500X	0.000	0.010	N.D.
	62500X	0.000	0.012	N.D.
0-2	50X	0.188	0.160	N.D.
	500X	0.048	0.050	N.D.
	2500X	0.000	0.018	N.D.
	12500X	0.000	0.013	N.D.
	62500X	0.000	0.009	N.D.
Group 1:				
1-1	50X	0.720	N.D.	N.D.
	500X	0.144 *	N.D.	N.D.
	2500X	0.018	N.D.	N.D.
	12500X	0.000	N.D.	N.D.
	62500X	0.000	N.D.	N.D.
1-2	50X	0.257 *	> 2.0 / > 2.0	> 2.0
	500X	0.062	0.976 / 1.263	> 2.0
	2500X	0.004	0.187 / 0.278 *	0.560
	12500X	0.000	0.023 / 0.062	0.132 *
	62500X	0.000	0.000 / 0.018	0.027
1-3	50X	0.213 *	> 2.0	N.D.
	500X	0.042	0.424 *	N.D.
	2500X	0.000	0.058	N.D.
	12500X	0.000	0.000	N.D.
	62500X	0.000	0.000	N.D.

1-4	50X	0.259 *	> 2.0 / > 2.0	> 2.0
	500X	0.050	1.882 / > 2.0	> 2.0
	2500X	0.002	0.348 / 0.506 *	0.886
	12500X	0.000	0.048 / 0.098	0.163 *
	62500X	0.000	0.000 / 0.037	0.039
1-5	50X	0.580	> 2.0 / > 2.0	1.616
	500X	0.111 *	1.774 / > 2.0	1.646
	2500X	0.010	0.336 / 0.471 *	0.313 *
	12500X	0.000	0.041 / 0.097	0.067
	62500X	0.000	0.000 / 0.030	0.021
1-6	50X	0.443	0.341	N.D.
	500X	0.161 *	0.191 *	N.D.
	2500X	0.026	0.071	N.D.
	12500X	0.000	0.025	N.D.
	62500X	0.000	0.016	N.D.

Group 2:

2-1	50X		> 2.0 / > 2.0	> 2.0
	500X		0.939 / 1.161	1.478
	2500X		0.161 / 0.200 *	0.280 *
	12500X		0.032 / 0.038	0.059
	62500X		0.016 / 0.017	0.022
2-2	50X		> 2.0 / > 2.0	> 2.0
	500X		> 2.0 / > 2.0	> 2.0
	2500X		1.092 / 1.316	1.158
	12500X		0.232 / 0.267 *	0.250 *
	62500X		0.050 / 0.063	0.061
2-3	50X		0.544	N.D.
	500X		0.121 *	N.D.
	2500X		0.028	N.D.
	12500X		0.010	N.D.
	62500X		0.013	N.D.
2-4	50X		> 2.0 / > 2.0	> 2.0
	500X		> 2.0 / > 2.0	> 2.0
	2500X		0.909 / 1.209	0.794
	12500X		0.177 / 0.232 *	0.156 *
	62500X		0.037 / 0.058	0.051
2-5	50X		1.860	> 2.0
	500X		0.379 *	0.836
	2500X		0.071	0.155 *
	12500X		0.018	0.030
	62500X		0.010	0.019
2-6	50X		> 2.0 / > 2.0	> 2.0
	500X		1.475 / 1.780	1.577
	2500X		0.333 / 0.383 *	0.357 *
	12500X		0.066 / 0.080	0.075
	62500X		0.019 / 0.078	0.025

Group 3:

3-1	50X	> 2.0
	500X	> 2.0
	2500X	> 2.0
	12500X	1.647
	62500X	0.362 *
3-2	50X	> 2.0
	500X	> 2.0
	2500X	1.032
	12500X	0.195 *
	62500X	0.053
3-3	50X	> 2.0
	500X	1.814
	2500X	0.312 *
	12500X	0.060
	62500X	0.026
3-4	50X	> 2.0
	500X	> 2.0
	2500X	0.895
	12500X	0.181 *
	62500X	0.048
3-5	50X	> 2.0
	500X	> 2.0
	2500X	> 2.0
	12500X	0.701
	62500X	0.146 *
3-6	50X	> 2.0
	500X	> 2.0
	2500X	> 2.0
	12500X	0.726
	62500X	0.172 *

@: Mouse serum diluted 50X, 500X, 2500X, 12500X and 62500X with 1% BSA.
#: Absorbance at 492nm.
*: End point of detectability.
$: N.D.: Assay not done because there was no serum for the assay.

In the second EIA, a rat anti-mouse:HRPO conjugate was added to the wells of a microtiter plate that had been coated with the following antigens or combinations of antigens: a.) NS5 (EN-80-1 antigen); b.) core antigen-envelope protein (EN-80-2 antigen); c.) partial core protein (EN-80-5 antigen); d.) NS5 and core antigen-envelope protein; e.) core antigen-envelope protein and partial core; and, f.) NS5, core antigen-envelope protein, and partial core. The samples used in the second EIA were as follows: 0-2 (50X diluted, from day 28); 0-2 (500X diluted, from day 28); 2-2 (2500X diluted, from day 28); 3-1 (12500X diluted, from day 39); 3-4 (2500X diluted, from day

39); 3-5 (2500X diluted, from day 39); 3-6 (2500X diluted, from day 39); and, 3-6 (12500X diluted, from day 39).

The results of the second EIA are shown below in Table 13.

Table 13

Sample ID	NS5	core-env	core	NS5 + core-env	NS5 + core	core + core-env	NS5 + core + core-env
Negative control:							
0-2 50X	0.018@	0.024	0.025	0.026	0.020	0.027	0.029
0-2 500X	0.008	0.010	0.011	0.014	0.014	0.022	0.019
Group II:							
2-2 2500X	0.004	0.398	0.007	0.489	0.009	0.313	0.388
Group III:							
3-1 12500X	0.002	0.506	0.009	0.760	0.009	0.513	0.472
3-4 2500X	0.003	0.220	0.007	0.344	0.006	0.192	0.227
3-5 2500X	0.003	0.705	0.007	1.168	0.006	0.592	0.747
3-6 2500X	0.005	0.693	0.005	1.012	0.008	0.542	0.704
3-6 12500X	0.005	0.144	0.008	0.224	0.009	0.126	0.134

@: Absorbance at 492nm.

Claims

1. A Hepatitis C virus (HCV)-derived composition comprising:

a) an isolated polypeptide comprising an HCV core antigen protein joined to an amino-terminal portion of an HCV envelope region in unprocessed form, wherein said amino-terminal portion of said HCV envelope region is sized such that said polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of said HCV; and

b) an isolated HCV nonstructural protein.

2. The composition of claim 1 wherein said isolated HCV nonstructural protein comprises an NS5 nonstructural protein.

3. The composition of claim 1 wherein said isolated HCV nonstructural protein comprises an NS3-NS4 unprocessed nonstructural protein.

4. The composition of claim 1 wherein said isolated polypeptide is produced by a prokaryotic host cell.

5. The composition of claim 1 wherein said isolated polypeptide is produced by a eukaryotic host cell that is unable to process said isolated polypeptide.

6. The composition of claim 1 or 2, wherein said isolated polypeptide and said nonstructural protein are bound to a solid substrate.

7. A method of making a composition comprising multiple polypeptides derived from a Hepatitis C virus (HCV), comprising the following steps:

a) introducing into a first host cell a first expression vector capable of expressing a nucleic acid molecule encoding an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region in unprocessed form, wherein said amino-terminal portion of said HCV envelope region is sized such that said polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of said HCV,

b) incubating said first host cell under conditions suitable for said expression vector to produce said polypeptide,

c) isolating said fusion polypeptide to provide an isolated fusion polypeptide, and

d) introducing into a second host cell a second expression vector capable of expressing a nucleic acid molecule encoding an isolated HCV nonstructural protein,

e) incubating said second host cell under suitable conditions for said nucleic acid molecule to produce said HCV nonstructural protein,

f) isolating said HCV nonstructural protein to provide an isolated HCV nonstructural protein, and then

g) combining said isolated fusion polypeptide and said isolated HCV nonstructural protein to form said composition.

8. A method of making a composition comprising multiple polypeptides obtained from a Hepatitis C virus (HCV), comprising the following steps:

a) introducing into a host cell an expression vector capable of expressing a first nucleic acid molecule encoding an isolated polypeptide comprising an HCV core antigen protein joined to an amino-terminal portion of an HCV envelope region in unprocessed form, wherein said amino-terminal portion of said HCV envelope region is sized such that said polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of said HCV, said expression vector further capable of expressing a second nucleic acid molecule encoding an HCV nonstructural protein,

b) incubating said first host cell under conditions suitable for said expression vector to produce said polypeptide and said HCV nonstructural protein, and

c) isolating said polypeptide and said HCV nonstructural protein.

9. A composition comprising an isolated polypeptide comprising a Hepatitis C virus (HCV) core antigen protein joined to an amino-terminal portion of an envelope region of said HCV, wherein said amino-terminal portion of said envelope region is sized such that said polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of said HCV, bound to a solid substrate.

10. An assay for the detection of Hepatitis C virus (HCV) in a sample, comprising:

a) providing an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein said amino-terminal portion of said HCV envelope region is sized such that said polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of said HCV,

b) contacting said isolated polypeptide with said sample under conditions suitable and for a time sufficient for said polypeptide to bind to one or more antibodies specific for said HCV present in said sample, to provide an antibody-bound polypeptide, and

c) detecting said antibody-bound polypeptide, and therefrom determining that said sample contains HCV.

11. The assay of claim 10 further comprising,

a) in step a), providing an HCV nonstructural protein bound to said solid substrate,

b) in step b), contacting said HCV nonstructural protein with said sample under conditions suitable and for a time sufficient for said HCV nonstructural protein to bind to one or more antibodies specific for said HCV present in said sample, to provide an antibody-bound HCV nonstructural protein, and

c) in step c), detecting one or both of said antibody-bound polypeptide or said antibody-bound HCV nonstructural protein, and therefrom determining that said sample contains HCV.

12. The assay of claim 11 wherein HCV nonstructural protein is selected from the group consisting of an NS5 nonstructural protein and an NS3-NS4 unprocessed nonstructural protein.

13. The assay of claim 11 or 12 wherein said sample is an unpurified sample.

14. The assay of claim 11 or 12 further comprising, prior to said contacting, the step of obtaining said sample from an animal.

15. The assay of claim 14 wherein said animal is a human being.

16. The assay of claim 11 or 12 wherein said assay is selected from the group consisting of a countercurrent immuno-electrophoresis (CIEP) assay, a radioimmunoassay, a radioimmunoprecipitation, an enzyme-linked immunosorbent assay (ELISA), a dot blot assay, an inhibition or competition assay, a sandwich assay, an immunostick (dip-stick) assays, a simultaneous assay, an immunochromatographic assay, an immunofiltration assay, a latex bead agglutination assay, an immunofluorescent assay, a biosensor assay, and a low-light detection assay.

17. The assay of claim 11 or 12 wherein said assay is not a western blot assay.

18. A method of producing an antibody, comprising the following steps:

a) administering to an animal an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein said amino-terminal portion of said HCV envelope region is sized such that said polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of said HCV under conditions suitable and for a time sufficient to induce an immune response in said animal to said polypeptide, thereby providing antibodies to said polypeptide, and

b) isolating said antibodies to said polypeptide.

19. Antibodies produced according to claim 18.

20. The antibodies of claim 19 wherein said antibodies are bound to a solid substrate.

21. An assay for the detection of Hepatitis C virus (HCV) in a sample, comprising:

a) contacting said sample with an antibody specific for an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein said amino-terminal portion of said HCV envelope region is sized such that said polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of said HCV, under conditions suitable and for a time sufficient for said antibody to bind said unprocessed HCV core antigen protein, to provide a bound antibody, and

b) detecting said bound antibody, and therefrom determining that said sample contains HCV.

22. The assay of claim 21 further comprising,

a) in step a), contacting said sample with a further antibody specific for an HCV nonstructural protein under conditions suitable and for a time sufficient for said further antibody to bind said HCV nonstructural protein, to provide a bound further antibody, and

b) in step b), detecting one or both of said bound antibody or said bound further antibody, and therefrom determining that said sample contains HCV.

23. A composition capable of eliciting an immune response in an animal comprising an isolated polypeptide comprising an unprocessed Hepatitis C virus (HCV) core antigen protein and an amino-terminal portion of an HCV envelope region, wherein said

amino-terminal portion of said HCV envelope region is sized such that said polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of said HCV, in combination with a pharmaceutically acceptable carrier or diluent.

24. The composition of claim 23 further comprising an HCV nonstructural protein.

25. A vaccine against Hepatitis C virus (HCV) comprising an immunoprotective amount of an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein said amino-terminal portion of said HCV envelope region is sized such that said polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of said HCV, in combination with a pharmaceutically acceptable carrier or diluent.

26. The vaccine of claim 25 further comprising an HCV nonstructural protein.

27. The vaccine of claim 25 or 26 wherein said vaccine is capable of eliciting an immune protective response in a human being.

28. The composition of any one of claims 1-9 or 23-24 for use as an active therapeutic substance.

29. The vaccine of any one of claims 25-27 for use as an active therapeutic substance.

30. The composition of any one of claims 1-9 or 23-24 for use in the manufacture of a medicament for inhibiting, preventing or treating HCV infection in an animal.

31. The vaccine of any one of claims 25-27 for use in the manufacture of a medicament for inhibiting, preventing or treating HCV infection in an animal.

32. A kit for the detection of Hepatitis C virus (HCV) comprising:

a) an isolated polypeptide comprising a Hepatitis C virus (HCV) core antigen protein joined to an amino-terminal portion of an envelope region of said HCV, wherein said amino-terminal portion of said envelope region is sized such that said

polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of said HCV, covalently bound to a solid substrate, and

b) one or both of a reagent or a device for detecting said isolated polypeptide.

33. The kit of claim 32 further comprising an HCV nonstructural protein and one or both of a reagent or a device for detecting said HCV nonstructural protein.

34. A kit for the detection of Hepatitis C virus (HCV) comprising:

a) an antibody specific for an isolated polypeptide comprising an unprocessed HCV core antigen protein and an amino-terminal portion of an HCV envelope region, wherein said amino-terminal portion of said HCV envelope region is sized such that said polypeptide has an epitopic configuration specific to an unprocessed core-envelope region of said HCV, and

b) one or both of a reagent or a device for detecting said antibody.

35. The kit of claim 34 further comprising a further antibody specific for an HCV nonstructural protein and one or both of a reagent or a device for detecting said further antibody.

Nucleotide Sequence

```
5'-ATGAGCACAAATCCTAAACCTCAAAGAAAAACCAAACGTAACACCAACCGCCGCCCACAG    60
   GACGTCAAGTTCCCGGGCGGTGGTCAGATCGTTGGTGGAGTTTACCTGTTGCCGCGCAGG   120
   GGCCCCAGGTTGGGTGTGCGCGCGACTAGGAAGACTTCCGAGCGGTCGCAACCTCGTGGA   180
   AGGCGACAACCTATCCCCAAGGCTCGCCGGCCCGAGGGCAGGACCTGGGCTCAGCCGGGG   240
   TACCCTTGGCCCCTCTATGGCAATGAGGGTCTGGGGTGGGCAGGATGGCTCCTGTCACCC   300
   CGAGGCTCTCGGCCTAGTTGGGGCCCCACGGACCCCCGGCGTAGGTCGCGTAATCTGGGT   360
   AAGGTCATCGATACCCTCACAGGTGGCTTCGCCGACCTCATGGGGTACATTCCGCTCGTC   420
   AGCGCCCCACTAGGAGGCGCTGCCAGGGCCCTGGGCCATGGCGTCCGGGTTCTGGAGGAC   480
   GGCGTGAACTATGCAACAGGGAATCTGCCCGGTTGCTCTTTCTCTATCTTCCTCTTAGCT   540
   TTGCTGTCTTGTTTGACCATCCCAGCTTCCGCTTACGAGGTGCGCAACGTGTCCGGGATA   600
   TACCATGTTACGAACGATTGCTCCAACTCAAGTATCGTGTATGAGGCAGCGGACATGATC   660
   ATGCACACC-3'                                                   669
```

Fig. 1A

Amino Acid Sequence

NH_2-MetSerThrAsnProLysProGlnArgLysThrLysArgAsnThr 15

AsnArgArgProGlnAspValLysPheProGlyGlyGlyGlnIle 30

ValGlyGlyValTyrLeuLeuProArgArgGlyProArgLeuGly 45

ValArgAlaThrArgLysThrSerGluArgSerGlnProArgGly 60

ArgArgGlnProIleProLysAlaArgArgProGluGlyArgThr 75

TrpAlaGlnProGlyTyrProTrpProLeuTyrGlyAsnGluGly 90

LeuGlyTrpAlaGlyTrpLeuLeuSerProArgGlySerArgPro 105

SerTrpGlyProThrAspProArgArgArgSerArgAsnLeuGly 120

LysValIleAspThrLeuThrGlyGlyPheAlaAspLeuMetGly 135

TyrIleProLeuValSerAlaProLeuGlyGlyAlaAlaArgAla 150

LeuGlyHisGlyValArgValLeuGluAspGlyValAsnTyrAla 165

ThrGlyAsnLeuProGlyCysSerPheSerIlePheLeuLeuAla 180

LeuLeuSerCysLeuThrIleProAlaSerAlaTyrGluValArg 195

AsnValSerGlyIleTyrHisValThrAsnAspCysSerAsnSer 210

SerIleValTyrGluAlaAlaAspMetIleMetHisThr-COOH 223

Fig. 1B

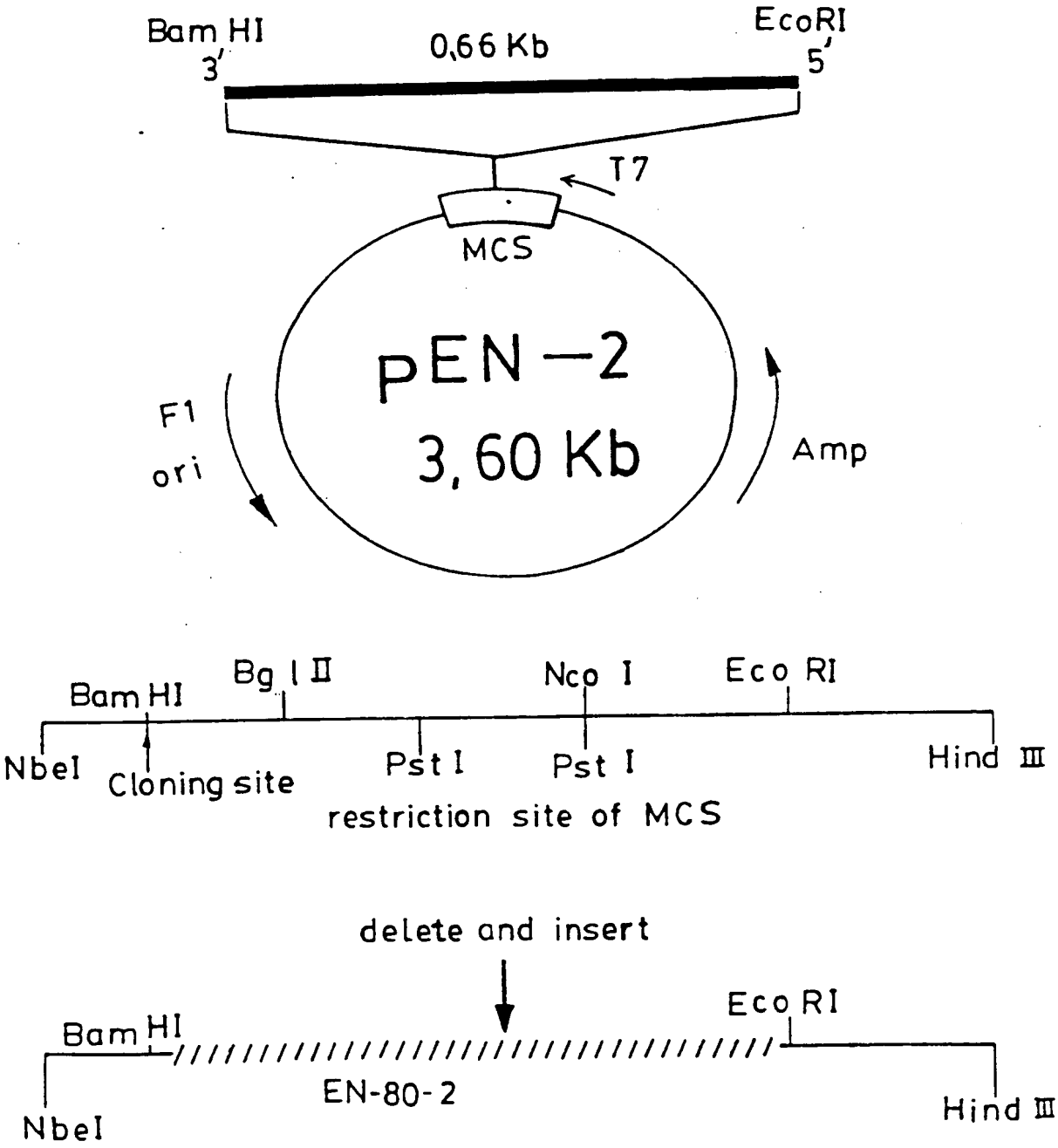

Fig. 2

Nucleotide Sequence

```
5'-GTGGAGGATGAGAGGGAAATATCCGTTGAGGCGGAGATCCTGCGTTTTTCCAGGAAATTC    60
   CCCCGGGCGATACCCATATGGGCCCGCCCGGATTACAATCCACCACTGATAGAGTCCTGG   120
   AAGGACCCGGACTATGTCCCCCCGGTGGTACACGGGTGCCCATTGCCACCTGCCAAGATC   180
   CCTCCAATACCACCTCCACGGAGGAAGAAGACGGTTGTCCTGACAGAGTCCGTCTATACT   240
   TCTGCCCTGGCGGACGTTGCTACAAAGACCTTCGGCAGCTCCGAGTCTACGCCCGTCGAC   300
   AGCGGCACAGCGACTGGCCTCCCGATCAACCTTCTGACGACGGCGACAAAGGGATCCGAC   360
   GTTGAGTCGTACTCCTCCATGCCCCCCCTCGAGGGAGAGCCAGGCGACCCCGATCTCAGC   420
   GACGGGTCTTGGTCTACTGTGAGCGTGGAGGCTAGTGAGGACGTTGTCTGCTGCTCGATG   480
   TCCTACACATGGACAGGCGCTTTAATCACGCCATGCGCTGCGGAGGAGAGCAAACTGCCC   540
   ATCAATGCGTTGAGCTTCTCTTTGTTGCGTCACCACAATATGGTCTACGCCACAACATCC   600
   CGCAGCGCAGACCAGCCGCAGAAAAAGGTCACCTTTGACAGACTGCAAGTCCTGGACGAC   660
   CACTACCGGGACGTACTCAAGGAGATGAAGGCGAAGGCGTCTACAGTTAAGGCTAAACTT   720
   CTATCCGTAGAAGAGGCCTGTAACGTGACGCCCCCACATTCGGCCAAATCCAAATTTGGC   780
   TACGGGGCGAAGGACGTCCGG-3'                                       801
```

Fig. 3A

Amino Acid Sequence

NH_2-ValGluAspGluArgGluIleSerValGluAlaGluIleLeuArg 15

PheSerArgLysPheProArgAlaIleProIleTrpAlaArgPro 30

AspTyrAsnProProLeuIleGluSerTrpLysAspProAspTyr 45

ValProProValValHisGlyCysProLeuProProAlaLysIle 60

ProProIleProProProArgArgLysLysThrValValLeuThr 75

GluSerValTyrThrSerAlaLeuAlaAspValAlaThrLysThr 90

PheGlySerSerGluSerThrProValAspSerGlyThrAlaThr 105

GlyLeuProIleAsnLeuLeuThrThrAlaThrLysGlySerAsp 120

ValGluSerTyrSerSerMetProProLeuGluGlyGluProGly 135

AspProAspLeuSerAspGlySerTrpSerThrValSerValGlu 150

AlaSerGluAspValValCysCysSerMetSerTyrThrTrpThr 165

GlyAlaLeuIleThrProCysAlaAlaGluGluSerLysLeuPro 180

IleAsnAlaLeuSerPheSerLeuLeuArgHisHisAsnMetVal 195

TyrAlaThrThrSerArgSerAlaAspGlnProGlnLysLysVal 210

ThrPheAspArgLeuGlnValLeuAspAspHisTyrArgAspVal 225

LeuLysGluMetLysAlaLysAlaSerThrValLysAlaLysLeu 240

LeuSerValGluGluAlaCysAsnValThrProProHisSerAla 255

LysSerLysPheGlyTyrGlyAlaLysAspValArg-COOH 267

Fig. 3B

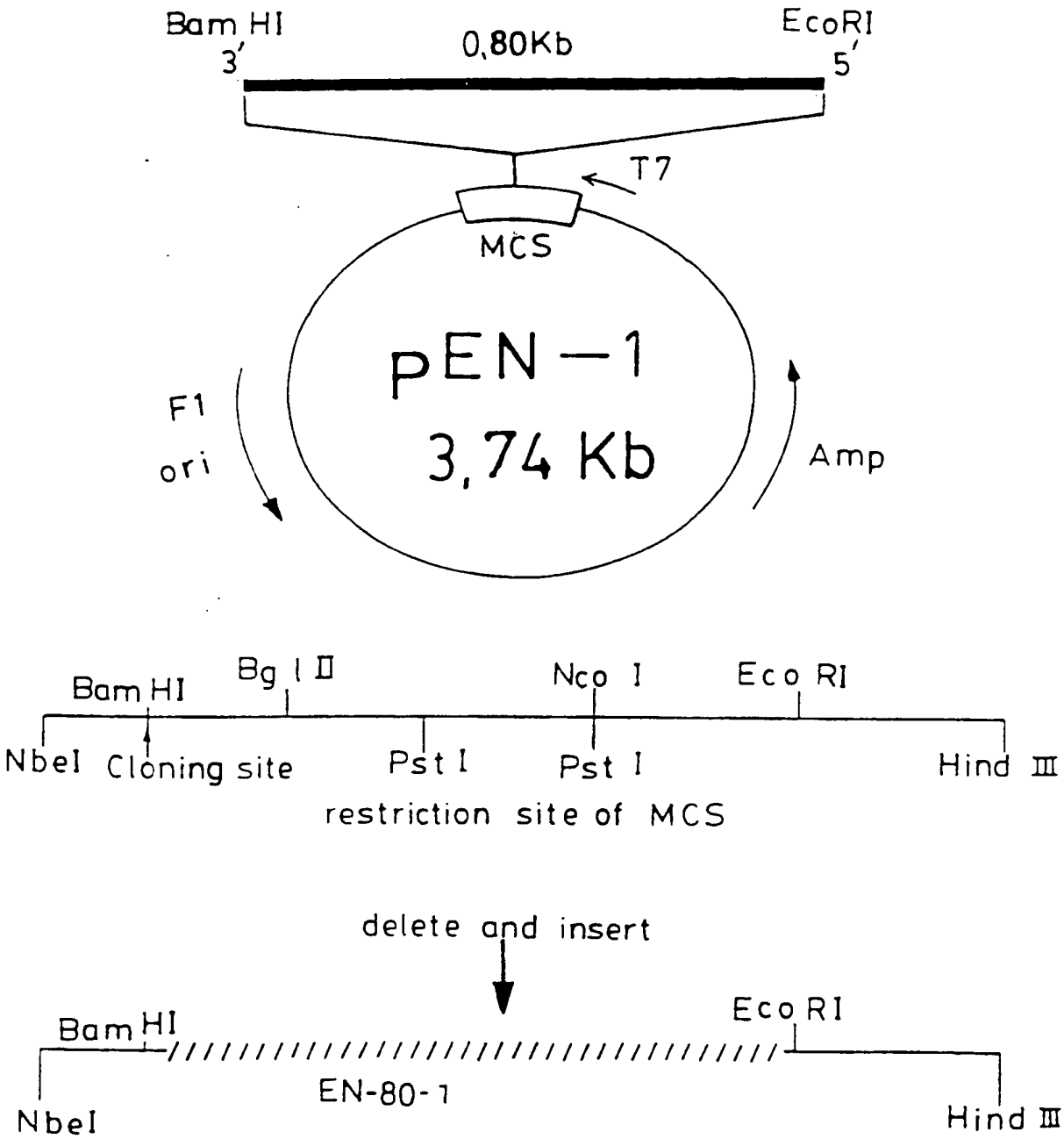

Fig. 4

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 96/07378

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07K14/18 C07K16/10 A61K39/29 G01N33/576

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 17110 (WELLCOME FOUNDATION) 2 September 1993 see the whole document ---	1-35
X	EP,A,0 463 848 (OSAKA UNIVERSITY) 2 January 1992 see the whole document ---	1-35
X	EP,A,0 450 931 (CHIRON) 9 October 1991 see the whole document ---	1-35
X	EP,A,0 442 394 (UNITED BIOMEDICAL) 21 August 1991 see the whole document ---	1-35
A	WO,A,94 25486 (LUCKY LIMITED) 10 November 1994 see the whole document -----	1-35

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 1996	28. 10. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

.formation on patent family members

Interr nal Application No
PCT/US 96/07378

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9425486	10-11-94	CN-A-	1122139	08-05-96
		JP-T-	8506833	23-07-96

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr nal Application No
PCT/US 96/07378

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		ZA-A-	9301203	04-10-93
EP-A-463848	02-01-92	AU-B-	652941	15-09-94
		AU-A-	7925691	02-01-92
		CA-A-	2045326	26-12-91
		JP-A-	7258290	09-10-95
		CN-A-	1059758	25-03-92
		AU-B-	650634	30-06-94
		AU-A-	6860891	02-01-92
		CA-A-	2045323	26-12-91
		CN-A-	1057861	15-01-92
		EP-A-	0464287	08-01-92
		JP-A-	6303980	01-11-94
		KR-B-	9512971	24-10-95
EP-A-450931	09-10-91	AT-T-	139343	15-06-96
		AU-B-	639560	29-07-93
		AU-A-	7651091	30-10-91
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		EP-A-	0693687	24-01-96
		ES-T-	2088465	16-08-96
		GB-A,B	2257784	20-01-93
		LT-A,B	1747	25-07-95
		LV-B-	10344	20-02-96
		WO-A-	9115771	17-10-91
EP-A-442394	21-08-91	US-A-	5106726	21-04-92
		CA-A-	2036463	17-08-91
		JP-A-	5148298	15-06-93
		KR-B-	9400755	29-01-94
		US-A-	5436126	25-07-95
		AU-B-	635124	11-03-93
		AU-A-	7439991	17-10-91
		AU-B-	646275	17-02-94
		CA-A-	2047792	27-01-92
		DE-T-	468527	24-02-94
		EP-A-	0468527	29-01-92
		ES-T-	2058049	01-11-94
		JP-A-	5222094	31-08-93